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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/11, C12Q 1/70, C07K 14/47, C12Q 1/68 — A1

(11) International Publication Number: WO 00/34466

(43) International Publication Date: 15 June 2000 (15.06.00)

(21) International Application Number: PCT/GB99/04116

(22) International Filing Date: 7 December 1999 (07.12.99)

(30) Priority Data:

9826888.1	7 December 1998 (07.12.98)	GB
09/282,210	31 March 1999 (31.03.99)	US

(81) Designated States: JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(54) Title: CERVICAL CANCER TREATMENT

(57) Abstract

A product that binds, causes a decrease in intracellular levels of, or inhibits the activity of Brn-3a for use in the treatment, prevention or diagnosis of a cervical cancer attributable to HPV. Methods of identifying such a product are also provided. The product may also be used to screen for individuals who are susceptible to cervical cancer.

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CERVICAL CANCER TREATMENT

Field of the invention

The invention relates to the diagnosis or treatment of cancer and to screening for individuals at risk from cancer.

Background to the invention

The ability of viruses to infect successfully an individual or to have other detrimental effects, such as causing oncogenesis, is determined by many factors. Such factors include the dose of virus which the host receives, the resistance of the host, the route of infection and the virulence of the virus.

In the case of certain viruses it has become clear that individuals within the same population can have different susceptibilities to the virus. Such differences can be caused by differences in levels of expression in host susceptibility factors. The finding of such host susceptibility factors and understanding the mechanism of their effect can be used to design therapeutic treatments against the virus or its detrimental effects.

Human papillomavirus types 16 and 18 (HPV-16 and HPV-18) are thought to play a part in causing cervical cancer in infected individuals. However HPV-16 and HPV-18 are found both in women with cervical cancer and in women with undetectable or minimal cervical abnormality. A polymorphism in a human protein has been shown to affect susceptibility to cervical cancer caused by HPV. Individuals with a particular polymorphism in the p53 tumour suppressor protein are found to have an increased risk of cervical cancer caused by HPV.

Summary of the invention

The inventors have found that the mean level of human transcription factor Brn-3a is over 300 fold higher in cervical intraepithelial neoplasia (CIN)lesions than in normal cervical material. Brn-3a is a member of the POU family of transcription factors and is expressed in neuronal cells and in cervical cells. The inventors also found that non-malignant cervical cells in the individuals with CIN lesions also had high levels of expression of Brn-3a. This latter finding suggested that the levels of

Brn-3a may not be important in determining whether a cervical cell became malignant or not. This work has now been published (1).

However the inventors have now shown that an antisense-based approach can be used to reduce levels of Brn-3a in a transformed cervical cell line, and that this causes a reversal of the malignant phenotype. In particular the cells exhibit a reduced cellular growth rate, a reduced saturation density and a reduced ability to grow in an anchorage independent manner.

The invention therefore provides a product that binds, causes a decrease in intracellular levels of, or inhibits the activity of Brn-3a for use in the treatment, prevention or diagnosis of a cervical cancer attributable to HPV.

Screens may be carried out to identify the product. Accordingly the invention further provides method of identifying a product that binds to Brn-3a comprising contacting Brn-3a, or a mimic of Brn-3a that can bind to a Brn-3a specific antibody, with a candidate substance and determining whether the candidate substance binds Brn-3a or the mimic of Brn-3a.

The invention also provides a method of identifying a product that causes a decrease in intracellular levels of Brn-3a comprising contacting a candidate substance with a cell or cell extract and determining whether the candidate causes a decrease in intracellular levels.

The invention further provides a method of identifying a product that inhibits the activity of Brn-3a comprising

(i) contacting Brn-3a, or a mimic of Brn-3a that can bind to a Brn-3a specific antibody, with a candidate substance and determining whether the candidate substance binds Brn-3a or the mimic of Brn-3a; or

(ii) contacting an agent that can bind to Brn-3a with a candidate substance and determining whether the candidate substance binds to the agent; or

(iii)(a) contacting Brn-3a, or a mimic of Brn-3a with Brn-3a activity, with a candidate substance under conditions that would permit activity of Brn-3a, and (b) determining whether the candidate substance inhibits the activity of Brn-3a or the mimic of Brn-3a.

As noted above the product may be used to treat cancer and therefore the

invention provides a method of treating a host suffering from cancer comprising administering to the host a therapeutically effective amount of the product.

Brief description of the drawings

Figure 1 shows results of reverse transcriptase/ polymerase chain amplification of either the Brn-3a (A) Brn-3b (B) or control cyclophilin (C) mRNAs in three samples obtained from women with no histologicaly detectable cervical abnormality (N) or three samples from CIN3 lesions (3). The positive control of cDNA prepared from human neuroblastoma mRNA (+) and the negative control of no cDNA (-) are also indicated.

Figure 2 shows Brn-3b mRNA levels in cervical samples from women with no histologicaly detectable abnormality (N), from CIN3 regions (CIN3) or from normal samples adjacent to a CIN3 lesion (N-CIN). The horizontal bar shows the mean in each group and the number of samples in each group is indicated (n).

Figure 3 shows Brn-3a mRNA levels in the same samples as in figure 2.

Figure 4 shows Brn-3b protein levels as determined by Western blotting of cervical samples from women with no histologicaly detectable abnormality (N), from CIN3 samples (CIN3) or from normal samples adjacent to a CIN3 lesion (N-CIN).

Figure 5 Brn-3a protein levels in the same samples as in Figure 4.

Figure 6 shows levels of Brn-3a protein in parental SiHa (panel a) or C-33 cells (panel b) cells or clonal cell lines transfected with expression vector lacking any insert, (Pci-neo) Brn-3a expression vector, (Brn-3a) Brn-3b expression vector (Brn-3b) or a vector expressing the antisense strand of the Brn-3a gene (-3A) and then grown in the presence or absence of dexamethasone (dex) Each data point shows a different independently isolated, clonal cell line.

Figure 7 shows levels of HPV protein in parental SiHa cells or clonal cell lines transfected with expression vector lacking any insert, (neo) Brn-3a expression vector (A), Brn-3b expression vector (B), or two different cell lines transfected with the Brn-3a anti sense vector (-3A clone 3 and -3A clone 5).

Figure 8 shows cell growth curves of SiHa cells. The growth rate of parental SiHa cells is compared to that of SiHa cells transfected with empty expression vector

(neo) Brn-3a expression vector, Brn-3b expression or the antisense Brn-3a vector (-3A) in the presence or absence or dexamethasone.

Figure 9 shows cell growth curves of C-33 cells. The growth rate of parental C-33 cells is compared to that of C-33 cells transfected with empty expression vector (neo), Brn-3a expression vector, Brn-3b expression or the antisense Brn-3a vector in the presence or absence of dexamethasone.

Figure 10 shoes saturation density of parental SiHa cells or SiHa – derived clones stably transfected with empty expression vector (neo), Brn-3a expression vector, Brn-3b expression vector or two cell lines(-3A clone 3 and -3A clone 5) transfected with the antisense Brn-3a vector and grown in the presence or absence of dexamethasone.

Figure 11 shows saturation density of parental C-33 cells or C-33 -derived clones stably transfected with empty expression vector (neo), Brn-3a expression vector, Brn-3b expression vector or the antisense Brn-3a vector (-3A) and grown in the presence or absence of dexamethasone.

Figure 12 shows anchorage independent growth as assayed by colony forming efficiency CFE (number of colonies formed/ number of colonies seeded X100). The result is show for parental SiHa cells and SiHa-derived clonal cell lines transfected with empty expression vector, Brn-3a expression vector, Brn-3b expression vector or the Brn-3b antisense vector (panel a) or for parental C-33 cells and similarly transfected stable cell lines derived from C-33 (panel b). Values are the mean of three separate determinations.

Figure 13 shows the volume of the tumour formed at the indicated day after injection of nude mice with SiHa cells stably transfected with empty expression vector (group 1, Gp1, open squares) or with SiHA cells stably transfected with the Brn-3a anti-sense construct (group 2, Gp2, solid diamonds). Values are the means of six injected animals whose standard deviation is shown by the bars.

Detailed description of the invention

Cancer Type

The cancer which is prevented, treated, diagnosed, or susceptibility to which

can be screened for, in the invention is a cervical cancer attributable to HPV. In particular the cancer is one in which the malignant state of the cell is maintained by the expression of HPV proteins in the cell, such as the HPV proteins E6 or E7. The expression of the HPV proteins is generally dependent on the presence of Brn-3a in the cell. Therefore, typically the cancer is one in which malignant cells comprise HPV virus or HPV genome. The HPV genome is generally integrated into the genome of the cell. The HPV is typically HPV-16 or HPV-18.

Typically the levels of Brn-3a are elevated in the malignant cell. A cervical cell which has elevated Brn-3a levels is one which has a higher level of Brn-3a than the average (mean) level of Brn-3a in the non-malignant cervical cells of females in a population sample. Typically in such cells the Brn-3a or Brn-3a mRNA levels are at least 10-, 20-, 50-, 100-, 300- or 500-fold those of the average levels. Typically in such cells the ratio of levels of Brn-3a mRNA to cyclophilin mRNA is greater than from 0.1:1, for example greater than 0.2, 0.4, 0.6 or 0.8:1.

The products of the invention

Any suitable product which binds Brn-3a may be used in the invention. Typically the product binds Brn-3a specifically. Such a product may or may not inhibit the activity of Brn-3a. The product may bind reversibly or irreversibly to Brn-3a. A product which binds irreversibly dissociates very slowly from Brn-3a because it would be very tightly bound, either covalently or non-covalently. Reversible binding, in contrast with irreversible binding, is characterised by a rapid dissociation of the Brn-3a/product complex.

The product may resemble a natural agent that binds Brn-3a either in its structure or binding characteristics. Such an agent is typically a cellular or HPV component that binds Brn-3a. In the case where the agent is a polynucleotide or a polypeptide the product may have homology with the natural agent. The product may bind Brn-3a at the same site as the agent binds. Such a product is typically able to compete for, or inhibit, the binding of the agent to Brn-3a.

In one embodiment the product does not bind Brn-3a at a site that overlaps with the site at which the agent binds. Typically such a product does not compete

with the agent for binding to Brn-3a; but may or may not inhibit the binding of the agent to Brn-3a. The product may or may not be able to bind Brn-3a at the same time as the agent binds Brn-3a. In one embodiment the product only binds Brn-3a when Brn-3a is bound to the agent.

Any suitable product that inhibits the activity of Brn-3a may be used in the present invention. Such a product may bind Brn-3a, typically with binding characteristics as discussed above. Typically the product inhibits the activity of Brn-3a in a specific or substantially specific manner.

The product may or may not cause a change in the structure of Brn-3a. In one embodiment the inhibitor of Brn-3a activity causes Brn-3a to change to a less active or non-functional form. The change may be reversible or irreversible. Typically Brn-3a only adopts such a changed form when bound to the product. An irreversible change may occur, for example, if Brn-3a is chemically modified or is broken down by the product, for example by the breaking of peptide bonds.

A product which binds and inhibits the activity of Brn-3a typically does so by (i) inhibiting the binding of Brn-3a to a cellular or HPV component which naturally binds Brn3a, and/or (ii) changing the structure of Brn-3a so that it has a decreased ability to 'activate' or direct a Brn-3a dependent effect mediated by the component.

In one embodiment the product inhibits the activity of Brn-3a in a cell not by binding Brn-3a, but by binding a cellular or HPV component. The product may bind and/or act on the component in the same manner as the product described above binds and acts upon Brn-3a. Thus the binding of the product to the component is typically specific and may be reversible or irreversible, and may or may not cause a change in the structure of the component. Such a product typically resembles the structure and/or binding characteristics of Brn-3a, and therefore the product may be act as a 'mimic' of Brn-3a which has a lower activity than Brn-3a. The product may bind the component at the same site, at an overlapping site or at a different site than the site at which Brn-3a binds.

The product may or may not compete with Brn-3a to bind the component. The product may or may not inhibit the binding of the component with Brn-3a. Typically the product binds the component without causing activation of the

component, or causes less activation than Brn-3a.

The product which binds the component may be a peptide which has homology with Brn-3a but which has no Brn-3a activity or a reduced Brn-3a activity. Typically the product has from 10%, 1%, 0.1% or less of the activity of Brn-3a.

The cellular component may be a co-activator protein which binds to Brn-3a, and typically increases the activity of Brn-3a.

Generally the HPV component is one which upon binding Brn-3a causes an increase in the expression of a factor which contributes or leads to the malignant phenotype of the cell. Typically the HPV component is the upstream regulatory region (URR) of the HPV genome. The component may comprise the ATGCAATT motif of the URR. This motif may play a role in the expression of the oncogenic HPV proteins E6 and E7.

Any suitable product that decreases intracellular levels of Brn-3a may be used in the present invention. Such a product typically inhibits the expression of Brn-3a or increases the breakdown of Brn-3a. Thus such a product causes a decrease in the expression and/or levels of Brn-3a in a cell when provided to a cell or inside a cell.

A product which inhibits the expression of Brn-3a generally inhibits one or more cellular or HPV components that promote the expression of Brn-3a, or stimulates one or more cellular or HPV components that inhibit the expression of Brn-3a. Typically these components are specific or substantially specific to the expression of Brn-3a. The product may bind and/or act on the component in the same manner as the product described above binds and acts upon Brn-3a. Thus the binding of the product to the component is typically specific and may be reversible or irreversible, and may or may not cause a change in the structure of the component. The component may directly or indirectly promote or inhibit the expression of Brn-3a.

Cellular components that directly promote expression include the promoter of Brn-3a, transcription factors that bind or affect expression from the Brn-3a promoter, an RNA polymerase that can express mRNA from the Brn-3a gene, nuclear factors that bind to Brn-3a mRNA and/or transport Brn-3a mRNA from the nucleus to the cytoplasm, translation factors that contribute to translating the Brn-3a mRNA to Brn-

3a protein, or factors that bind and/or transport Brn-3a protein into the nucleus.

Components that indirectly promote expression include components that are one step removed from the Brn-3a expression pathway, such as the promoters, transcription factors, polymerases, nuclear factors, translation factors of components that directly promote the expression of Brn-3a. Components that indirectly promote expression may thus be one, two or more steps removed from the components that directly promote expression of Brn-3a.

Thus the product may inhibit transcription or translation of Brn-3a. Preferably the product is a specific inhibitor of transcription from the Brn-3a gene, and does not inhibit transcription from other genes. The product may bind to the Brn-3a gene either (i) 5' to the coding sequence, and/or (ii) to the coding sequence, and/or (iii) 3' to the coding sequence. Thus the product may bind to the Brn-3a promoter, and inhibit the initiation of transcription. As discussed above the product may bind and inhibit the action of a protein which is required for transcription from the Brn-3a gene.

The product may bind to the untranslated or translated regions of the Brn-3a mRNA. This could prevent the initiation of translation. Alternatively the inhibitor could bind to a protein which associates with the untranslated region and prevent the protein associating with the untranslated region.

Products which are polynucleotides, such as the antisense polynucleotides discussed below, may be chemically modified. This may enhance their resistance to nucleases and may enhance their ability to enter cells. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-

O-alkyloligoribonucleotides.

Antisense inhibitors

As noted above, the expression of Brn-3a in a cell may be reduced by the presence in that cell of a product which can bind to the Brn-3a mRNA. Therefore a polynucleotide which is capable of hybridizing to Brn-3a mRNA can constitute an appropriate inhibitor of Brn-3a expression.

The polynucleotide may be antisense to the Brn-3a mRNA. Such a polynucleotide may be capable of hybridising to Brn-3a mRNA and may thus inhibit the expression of Brn-3a by interfering with one or more aspects of Brn-3a mRNA metabolism including transcription, mRNA processing, mRNA transport from the nucleus, translation or mRNA degradation. The antisense polynucleotide may be DNA, but is typically RNA. The antisense polynucleotide may be provided as single or double stranded polynucleotide. The antisense polynucleotide typically hybridises to the Brn-3a mRNA to form a duplex (typically an RNA-RNA duplex) which can cause direct inhibition of translation and/or destabilisation of the mRNA. Such a duplex may be susceptible to degradation by nucleases.

The antisense polynucleotide may hybridize to all or part of the Brn-3a mRNA. Typically the antisense polynucleotide hybridizes to the ribosome binding region or the coding region of the Brn-3a mRNA. The polynucleotide may be complementary to all of or a region of the Brn-3a mRNA. For example, the polynucleotide may be the exact complement of all or a part of Brn-3a mRNA. However, absolute complementarity is not required and polynucleotides which have sufficient complementarity to form a duplex having a melting temperature of greater than 20°C, 30°C or 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to the Brn-3a mRNA under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

It is preferred that the polynucleotide hybridizes to all or part of the region of the Brn-3a mRNA corresponding to the coding sequence defined by nucleotides 1 to

1272 of SEQ ID NO:1. In one preferred embodiment the antisense polynucleotide sequence is complementary to the entire coding sequence of the mRNA and to the 50 nucleotides of the mRNA immediately 5' of the coding sequence. However, the polynucleotide may hybridise to all or part of the 5'- or 3'-untranslated region of the mRNA. The polynucleotide will typically be from 6 to 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length. The polynucleotides may be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 1000, 2000 or 3000 or more nucleotides in length. In one embodiment the length of the antisense oligonucleotide is the same as SEQ ID NO:1 or up to a few nucleotides, such as five or ten nucleotides, shorter than SEQ ID NO: 1.

Substances that provide the product as discussed above

The invention may be carried out by administering a substance which provides a product with any of the above properties *in vivo*. Such a substance is also included in the term 'product'. Typically the substance provides the product extracellularly or intracellularly, such as in the cytoplasm or nucleus. In one embodiment the substance provides the product only in a malignant cell or in a cell with the characteristics of a malignant cell, such as a cell with a high rate of cell division. The substance may provide the product in a tissue specific manner, such as only in cervical cells. The substance may provide the product in the region of a malignant or cervical cell.

Typically the substance is an inactive or precursor form of the product which can be processed *in vivo* to provide the product. Thus the substance may comprise the product associated, covalently or non-covalently, with a carrier. The substance can typically be modified or broken down to provide the product. As discussed below the substance may, for example, be a polynucleotide which is processed, for example transcribed and/or translated to provide a product as discussed above.

Use of the product

A product which binds Brn-3a or Brn-3a mRNA can be used to treat, prevent

or diagnose cancer or to determine whether an individual is susceptible to cancer.

Screening to diagnose cancer or determine susceptibility to cancer

The finding of elevated levels of Brn-3a or Brn-3a mRNA in a cell suggest that the cell is a malignant cell or is at risk of becoming a malignant cell. Measuring the levels of Brn-3a or Brn-3a mRNA in a cell can therefore be used to diagnose cancer or to determine susceptibility to cancer. The levels of Brn-3a or Brn-3a mRNA may be measured in vivo or in vitro. Thus the invention provides a means for measuring the level of Brn-3a or for typing individuals who are predisposed to expressing elevated levels of Brn-3a or Brn-3a mRNA for use in a method of diagnosis of cervical cancer or determining susceptibility to cervical cancer.

The invention also provides a method of diagnosing cervical cancer in a female or identifying a female who is susceptible to cervical cancer comprising determining whether cervical cells taken from the female express, or are predisposed to expressing, elevated levels of (i) Brn-3a, or (ii) Brn-3a mRNA.

A product which binds to Brn-3a or Brn-3a mRNA can be used to measure levels of Brn-3a or Brn-3a mRNA in a cell. Generally such a product is labelled directly, or can be labelled indirectly.

Typically the product is used to measure the levels of Brn-3a or Brn-3a mRNA in cervical cells of a female human being. Such cervical cells are obtained from the female, typically as a cervical smear. Thus females in a population can be screened to diagnose cervical cancer or determine susceptibility to cervical cancer.

The presence of HPV virus may also be determined in such screen.

Use of a product that binds Brn-3a or Brn-3a mRNA in therapy.

The accumulation in cells of a product that binds Brn-3a or Brn-3a mRNA can be used as a marker for cells in which Brn-3a levels are elevated. Thus the product can be used to direct an agent or effect to a cell in which the expression of Brn-3a is elevated. The agent or effect may be therapeutic to the cell, typically contributing to the reversal of a malignant phenotype. Such an agent may bind to the product and typically inhibits the activity of Brn-3a if the product is also bound to

Brn-3a. The agent or effect may be detrimental to the cell, typically inhibiting the growth or replication of the cell, or killing the cell. The agent is typically a toxin. The effect is typically electromagnetic radiation.

A product that inhibits the expression of, causes a decrease in intracellular levels of or inhibits the activity Brn-3a can be used to prevent or treat the cancers discussed above.

Identifying a product that causes a decrease in intracellular levels of Brn-3a

The invention provides a method of identifying a product that causes a decrease in the intracellular levels of Brn-3a comprising providing a candidate substance to a cell or cell extract and determining whether the candidate causes a decrease in intracellular levels. The cell may be any of the mammalian cells discussed herein which express Brn-3a.

Identifying a product that inhibits expression of Brn-3a

A product that decreases intracellular levels of Brn-3a may do so by inhibiting the expression of Brn-3a. Thus the invention provides a method of identifying a product that inhibits expression of Brn-3a comprising providing a candidate substance to one or more components of the intracellular expression pathway of Brn-3a, or functional analogues of these components, and determining whether

(i) the candidate substance binds or inhibits component(s) that promote the expression of Brn-3a; or

(ii) the candidate substance stimulates component(s) that inhibit the expression of Brn-3a.

The term 'component' includes the natural component or a functional analogue of the component.

Thus the product may be identified by providing a candidate substance to the component and determining whether the candidate substance binds the component. Any suitable binding assay format can be used, such as the formats discussed below.

In another embodiment the product is identified by providing a candidate

substance to the component under conditions that permit activity of the component, and determining whether the candidate substance inhibits or stimulates the activity of the component.

Typically the component of the cellular expression pathway used in the method is specific or substantially specific to the expression of Brn-3a. Typically in the method one or more of the following components are used: a Brn-3a promoter, transcription factors that bind or affect expression from the Brn-3a promoter, an RNA polymerase that can express mRNA from the Brn-3a gene, nuclear factors that bind to Brn-3a mRNA and/or transport Brn-3a mRNA from the nucleus to the cytoplasm, translation factors that contribute to translating the Brn-3a mRNA to Brn-3a protein, or factors that bind and/or transport Brn-3a protein into the nucleus.

Functional analogues of any of the above components may be used in the method. The analogues will have some or all of the relevant activity of the natural component. Typically the analogues comprise fragments of the natural components. In the case of components which are polynucleotides or polypeptides the analogues generally have homology with the natural component.

The components may be provided from a cell. Thus the components may be inside a cell, typically a recombinant or natural cell in which the components are recombinantly or naturally expressed. The components may be provided in the form of a cell extract or may be purified, or partially purified, from a cell extract. Typically the components are in or from a human cell, for example one which expresses Brn-3a, such as a neuronal cell or cervical cell.

The cell may be a mammalian cell, such as a primate or rodent cell, for example a mouse or rat cell. The cell may comprise an HPV genome, typically integrated into the genome of the cell. The cell may be malignant or normal.

Cellular or HPV components of the Brn-3a expression pathway are known or can be readily obtained by the skilled person. They can, for example, be purified from cells based on their ability to bind Brn-3a or Brn-3a mRNA.

Products which inhibit transcription Brn-3a mRNA

Products which inhibit transcription of Brn-3a can be identified in a method

comprising

(i) providing a test construct comprising a first polynucleotide sequence with Brn-3a promoter activity operably linked to a second polynucleotide sequence to be expressed in the form of mRNA;

(ii) contacting a candidate substance with the test construct under conditions that would permit the second polynucleotide sequence to be expressed in the form of mRNA in the absence of the substance; and

(iii) determining whether the substance inhibits expression from the construct.

Products which inhibit transcription of Brn-3a mRNA may be also identified in a method comprising

(i) providing a test construct comprising a polynucleotide sequence with Brn-3a promoter activity operably linked to a coding sequence;

(ii) contacting a candidate substance with the test construct under conditions that would permit the polypeptide encoded by the coding sequence to be expressed in the absence of the substance; and

(iii) determining whether the substance inhibits expression from the construct.

The polynucleotide with Brn-3a promoter activity may comprise:

(i) the sequence of a human or animal Brn-3a promoter;

(ii) a sequence which has homology with (i); or

(iii) a sequence which is a fragment of (i) or (ii).

The sequence (i) is generally a mammalian Brn-3a promoter, such as a primate or a rodent, typically a mouse or rat, Brn-3a promoter. Generally (i) comprises at least from nucleotides -500 to -1, typically -300 to -1 of the Brn-3a gene (the numbers being relative to the transcription start site).

Typically the polynucleotide comprises the sequences present in (i) which bind transcription factors or the RNA polymerase, or instead of any of these sequences homologues of the sequences able to bind the same transcription factors and RNA polymerase. Typically such sequences or their homologues are present in the polynucleotide in the same order and/or substantially the same relative spacing as in (i).

Generally this method is carried out in conditions which in the absence of the

test compound lead to expression of the coding sequence from the nucleic acid. The nucleic acid may also comprise other untranscribed or untranslated regions of the Brn-3a gene. The coding sequence typically encodes a protein that is able to act as a reporter of expression. The assay may be carried out in a cell which harbours the nucleic acid. The substance may be tested with any other known promoter to test the possibility that the test substance is a general inhibitor of gene expression.

Any reporter polypeptide may be used, for example luciferase, GUS or GFP. Luciferase is assayed by detecting chemiluminescence. GUS is assayed by measuring the hydrolysis of a suitable substrate, for example 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid (X-gluc)or 4-methylumbelliferyl-β-glucuronide (MUG). The hydrolysis of MUG yields a product which can be measured fluorometrically. GFP is quantified by measuring fluorescence at 590nm after excitation at 494nm. These methods are well known to those skilled in the art.

Alternatively the coding sequence may be the Brn-3a coding sequence itself, or a fragment of this sequence. The expression of the Brn-3a may be measured by for example, Northern/RNA blotting, Western/antibody blotting, RNA in situ hybridization or immunolocalisation.

Identifying a product that binds to or inhibits the activity of Brn-3a

The invention provides a method of identifying products that bind, and which may also inhibit the activity of, Brn-3a based on the ability of the product to bind Brn-3a. Any suitable format may be used for determining whether a product is capable of binding Brn-3a.

Thus the invention provides a method of identifying a product that binds to Brn-3a comprising providing Brn-3a, or a mimic of Brn-3a that can bind to a Brn-3a specific antibody, to a candidate substance and determining whether the candidate substance binds Brn-3a or the mimic of Brn-3a.

Brn-3a for use in the method can be obtained by known techniques. The nucleotide sequence encoding Brn-3a is provided herein as SEQ ID NO:1. This sequence information can be used by the skilled man to produce Brn-3a protein using routine methods.

The Brn-3a or the mimic is typically

(i) human Brn-3a;

(ii) a homologue of (i); or

(iii) a fragment of (i) or (ii).

The mimic may, however, be a mimic which is not derived from Brn-3a. Such a mimic may have been designed (e.g. computationally) to resemble Brn-3a in its binding characteristics and/or may have been selected (e.g from a library of substances) based on its ability to bind agents which bind Brn-3a.

In the method Brn-3a or the mimic is generally in a suitable buffer, which includes any suitable biological buffer that can provide buffering capability at a pH conducive to the reaction requirements of the Brn-3a. The Brn-3a may be in conditions, including temperatures, which are similar to intracellular conditions.

In the method the Brn-3a or mimic may be inside a cell or outside a cell. The cell may be the cell in which the Brn-3a naturally occurs, or a cell in which the Brn-3a or mimic is expressed recombinantly. The cell may be treated with agents which permeabilise the cell surface allowing test substances to enter the cell more readily. The Brn-3a or mimic may be in the form of an extract from such cells.

Binding assays

Methods which determine whether a candidate substance is able to bind the Brn-3a may comprise providing Brn-3a or the mimic to a candidate substance and determining whether binding occurs, for example by measuring the amount of the candidate substance which binds Brn-3a. The binding may be determined by measuring a characteristic of the Brn-3a that changes upon binding, such as spectroscopic changes.

The assay format may be a 'band shift' system. This involves determining whether a test candidate advances or retards Brn-3a on gel electrophoresis relative to Brn-3a in the absence of the compound

The method may be a competitive binding method. This determines whether the candidate is able to inhibit the binding of Brn-3a to an agent which is known to bind to the Brn-3a, such as an antibody specific for Brn-3a.

In such a method the candidate substance may inhibit the binding of the agent to Brn-3a either by the candidate substance binding Brn-3a or by the candidate substance binding the agent. Thus a competitive inhibition method can identify not only substances that bind Brn-3a, but also substances that act as mimics of Brn-3a.

The competitive binding method may comprise

(i) incubating the Brn-3a with the candidate substance and a labelled reference compound that is known to bind the product;

(ii) determining the amount of the labelled reference compound that is bound to the product; and

(iii) comparing the amount of bound labelled reference compound determined in step (ii) with the amount of said compound that binds to the product in the absence of the candidate substance;

wherein any reduction in the binding of the labelled reference compound in the presence of the candidate substance compared to the binding in the absence of the candidate substance shows that the candidate substance binds either the product or the reference compound and thus may be an inhibitor of Brn-3a activity.

The amount of the labelled reference compound bound to the Brn-3a may be measured directly or indirectly. A direct measurement may be carried out by removing assay mixture containing the unbound labelled reference compound and measuring the amount of label that is in the product fraction. Alternatively, the amount of labelled reference compound bound to the product could be determined indirectly by measuring the amount of label remaining in the assay solution after removal of the product fraction, which will be inversely related to the amount that has bound to the product.

In a competitive binding assay system, the Brn-3a may be immobilised on a solid support or may be in solution. The use of immobilised product has the advantage that, after the binding reaction is complete, the product/labelled reference compound complex may be separated from the labelled reference compound that remains in solution by simply removing the solution away from the solid support. If, on the other hand, the product is not immobilised during the assay but rather is in solution, then it will generally be necessary to devise a means for separating the

product/labelled reference compound complex from the uncomplexed reference compound before measuring the amount of label. Such separation could be achieved, for example, by precipitating the product using an antibody to the product or by using a non-specific precipitation technique.

The different types of assays mentioned above can be used to measure binding between any two substances mentioned herein.

Characteristics of the products

A product that inhibits Brn-3a expression and/or activity is one which produces a measurable reduction in Brn-3a expression and/or activity in the methods described above. Preferred products are those which inhibit Brn-3a expression and/or activity by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the product of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1} or 100mg ml^{-1}. The percentage inhibition represents the percentage decrease in expression/activity in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition and concentration of inhibitor may be used to define an inhibitor of the invention, with greater inhibition at lower concentrations being preferred. The inhibitor may or may not be toxic towards humans or animals.

Candidate substances which show activity in assays such as those described herein can then be further tested, for example on malignant cell or in animals with cancer.

Products of the invention may be present in a substantially isolated form. It will be understood that the product may be mixed with carriers or diluents which will not interfere with the intended purpose of the product and still be regarded as substantially isolated. A product of the invention may also be in a substantially purified form, in which case it will generally comprise more than 90%, e.g. 95%, 98% or 99% of the polypeptide, polynucleotide or dry mass of the preparation.

Labels

Suitable labels for use in the methods or assays described herein include radioisotopes, e.g. ^{125}I, ^{35}S, ^{32}P enzymes, antibodies, polynucleotides and polypeptides such as biotin.

Methods of making antibodies

Antibodies to any of the substances discussed herein can be produced by use of the following methods. An antibody to the substance may be produced by raising antibody in a host animal against the whole substance or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known.

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody

obtained may be isolated and, if desired, purified.

Homologues

Homologues of polynucleotides or polypeptides are referred to herein. Typically a polynucleotide or polypeptide which is homologous to another polynucleotide or polypeptide is at least 70% homologous to the polynucleotide or polypeptide, preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto. Such homology may exist over a region of at least 15, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring polynucleotide or polypeptide homology are well known in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395).

Candidate substances

Suitable candidate substances which tested in the above methods include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies) which are specific for Brn-3a or mimics of Brn-3a. Furthermore, combinatorial libraries, defined chemical identities, peptide and peptide mimetics, oligonucleotides and natural product libraries , such as display libraries (e.g. phage display libraries) may also be tested. The candidate substances may be chemical compounds. Batches of the candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substances of batches which show inhibition tested individually.

Mimics

A mimic of any of the polypeptides which are discussed herein(e.g. the HPV or cellular components) is typically a polypeptide with homology to the original polypeptide. Similarly a mimic of any of the polynucleotides discussed herein is typically a polynucleotide with homology to the original polynucleotide. However the mimics may be polypeptides or polynucleotides which are not homologous, or

can be non-polypeptide or non-polynucleotide substances.

Typically a mimic of a substance binds a specific antibody which is able to bind the substance. The mimic typically has a shape, size, flexibility or electronic configuration which is substantially similar to the original substance. It is typically a derivative of the original substance.

Administration of polynucleotides

As discussed above products of the invention may be expressed from polynucleotides *in vivo*, particularly in the case of products which are antisense polynucleotides. Thus typically the product is expressed in a cell from a recombinant replicable vector. Such a replicable vector comprises a polynucleotide which when transcribed gives rise to product.

Thus the product may be provided by delivering such a vector to the cell and allowing transcription from the vector to occur. Such a vector is understood to be a 'product' of the invention. Generally on the vector the polynucleotide giving rise to the product is operably linked to a control sequence which is capable of providing for the transcription of the polynucleotide giving rise to the product. The term 'operably linked' refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence 'operably linked' to a sequence giving rise to the product is ligated in such a way that transcription of the sequence is achieved under conditions compatible with the control sequences.

The vector may be for example, a plasmid or virus vector provided with an origin of replication, optionally a promoter for transcription to occur and optionally a regulator of the promoter. The vector may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used in vitro, for example for the production of the product or used to transfect or transform a host cell. The term 'host cell' refers to a cell of any of the cancers discussed above. It may be a malignant or normal cell. The vector may also be adapted to be used in vivo, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as b-actin promoters, may be used. Tissue-specific promoters, in particular cervical or epithelial cell specific promoters (for example the Brn-3a involucin or keratin promoters), are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). All these promoters are readily available in the art.

The vector may further include sequences flanking the polynucleotide giving rise to antisense RNA which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences can be used to prepare a viral vector suitable for delivering the polynucleotides of the invention to a mammalian cell. Other examples of suitable viral vectors include herpes simplex viral vectors (for example as disclosed in WO 98/04726 and WO 98/30707) and retroviruses, including lentiviruses, adenoviruses, adeno-associated viruses and HPV viruses (such as HPV-16 or HPV-18). Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

Administration

The vectors and antisense oligonucleotides of the invention, optionally with an additional therapeutic polypeptide or nucleic acid/vector encoding said therapeutic polypeptide, may thus be administered to a human or animal in need of treatment.

Cancers which may be treated using the vectors, viral strains, antisense oligonucleotides and compositions of the invention cervical cancers attribute to HPV. The condition of a patient suffering from such a cancer can thus be improved.

The antisense oligonucleotides and compositions comprising antisense oligonucleotides of the invention together may be administered by direct injection into the site to be treated, for example cervical tissue. Preferably the antisense oligonucleotides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, or transdermal administration.

The dose at which an antisense oligonucleotide is administered to a patient will depend upon a variety of factors such as the age, weight and general condition of the patient, the cancer that is being treated and the stage which the cancer has reached, and the particular antisense oligonucleotide that is being administered. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight.

The polynucleotides giving rise to the product of the invention *in vivo* may be administered directly as a naked nucleic acid construct. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam^{TM} and transfectam^{TM}).

Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition. Preferably the naked nucleic acid construct, viral vector comprising the polynucleotide or composition is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, or transdermal administration.

The pharmaceutical composition is administered in such a way that the

polynucleotide of the invention, viral vector for gene therapy, can be incorporated into cells at an appropriate area. When the polynucleotide of the invention is delivered to cells by a viral vector, the amount of virus administered is in the range of from 10^6 to 10^{10} pfu, preferably from 10^7 to 10^9 pfu, more preferably about 10^8 pfu for adenoviral vectors. When injected, typically 1-2 ml of virus in a pharmaceutically acceptable suitable carrier or diluent is administered. When the polynucleotide of the invention is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 μg to 10 mg.

Where the polynucleotide giving rise to the product is under the control of an inducible promoter, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the polypeptide of the invention ceases. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The use of tissue-specific promoters will be of assistance in the treatment of disease using the polypeptides, polynucleotide and vectors of the invention. It will be advantageous to be able express therapeutic genes in only the relevant affected cell types, especially where such genes are toxic when expressed in other cell types.

The routes of administration and dosages described above are intended only as a guide since a skilled physician will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

Human use

Products found to bind, cause a decrease in intracellular levels of, or inhibit the activity of Brn-3a in the screening procedures described above may be used to treat the cancers discussed above. The condition of a patient suffering from such cancer can therefore be improved by administration of such a product. A therapeutically effective amount of such an product may be given to a human patient in need thereof.

The formulation of the product for use in preventing or treating the cancer

will depend upon factors such as the nature of the substance identified, whether a pharmaceutical or veterinary use is intended, etc. Typically the product is formulated for use with a pharmaceutically acceptable carrier or diluent. For example it may be formulated for topical, parenteral, intravenous, intramuscular, subcutaneous, transdermal or oral administration. A physician will be able to determine the required route of administration for each particular patient. The pharmaceutical carrier or diluent may be, for example, an isotonic solution.

The dose of product may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight. Again, a physician will be able to determine the required route of administration and dosage for any particular patient.

The following Examples illustrate the invention:

Example 1

Materials and Methods

Cervical biopsies, plasmids and HPV-16 DNA

Human cervical biopsies were obtained from women attending the Colposcopy Clinic, Whittington Hospital, Highgate Hill, UK. who were referred with abnormal cervical smears ranging from mild to severe dyskariosis. All women provided informed consent at the time of colposcopy. Ethical permission was obtained from the Whittington Hospital Ethical Committee following review of the study protocol.

Samples to be tested were chosen to represent a range of histologic grades independent of HPV status. Biopsy was either by punch or loop excision. Each specimen was divided into 1mm segments. Alternate sections of the biopsies were either transported in liquid nitrogen and stored at -70°C before nucleic acid extraction or were sent for histology. Sections were histologically classified in respect of the presence of cervical intraepithelial neoplasia. High grade lesions were those with a diagnosis of CIN2/3 and low grade of CIN1. Normal tissues were also classified.

The biopsies were made up of 1) 14 CIN3 lesions (CIN3); 2) 16 normal samples from regions adjacent to CIN3 lesions (N-CIN); and 3) 16 normal samples from individuals with no histologically detectable cervical abnormality. Complementary DNA (cDNA) obtained from human neuroblastoma cell lines expressing Brn-3a and Brn-3b was used as positive controls for RT-PCR experiments. Water only or human fibroblast mRNA (which does not express Brn-3a or Brn-3b) was used as a negative control. Plasmids with human Brn-3a and Brn-3b cDNA were used as positive controls for polymerase chain reaction (PCR) experiments. HPV-16 DNA was used as positive control for subsequent HPV PCR procedures, with water or DNA or RNA from an HPV-negative neuroblastoma cell line being used as negative controls.

RNA and DNA extractions from human cervical biopsies and Reverse Transcriptase (RT)-PCR

RNA was prepared using the guanidinium isothiocyanate method (2). The samples of about 33mg were homogenised in 250µl denaturation solution containing 4M guanidinium thiocyanate, 25mM sodium citrate, pH7, 0.1M 2-mercaptoethanol, 0.5% N-Laurolsarcosine. DNA was extracted using guanidinium isothiocyanate as previously described (3). Resultant RNA and DNA were respectively treated with 250mg/ml DNase and RNase. About 0.1µg of the resultant RNA from each sample was used as a template for cDNA synthesis. The synthesised cDNA was used in RT-PCR assays as previously described (4, 5) using the following oligonucleotide primers:-

Brn-3a: 5'GTCGACATGGACTCGGACACG-3', 3'-ACGGTGAATGACTCCCCCGA-5';

Brn-3b: 5'-GGAGAAGAAGCGCAAGC-3', 3'CTGAGAACCGGAGAGGTCT-5'.

The amplification of the invariantly expressed human cyclophilin mRNA used as a control was carried out in parallel using the following primers: 5'-TTGGGCCGCGGTACTCCTTTCA-3', 3'-TTTCGTATGGCCCAGGACCG-5' (4). HPV-16 DNA and cDNA prepared from HPV-16 mRNA was amplified using a hot-start of 95°C and annealing temperature of 54°C for 35 cycles with the

following primers: 5'-gagaagcttCTGCAATGTTTCAGGACCC-3', 3'-gactcgaGTGCCCATTAACAGGTCTTC-5' (3).

In all cases, 20µl of each PCR product were fractionated on a 2% agarose gel and blotted onto Hybond-N+ nylon membrane (Amersham, UK) and hybridised with homologous complimentary ^{32}P-labelled probes. Membranes were exposed to Kodak films and the subsequent autoradiographs were then analysed using a densitometer (BioRad, Hercules). We have previously shown that this blotting procedure, in conjunction with the RT/PCR conditions used, allows the accurate quantifying of the Brn-3a and Brn-3b mRNAs relative to the constitutively expressed cyclophilin mRNA (4-8).

Western blotting

Samples for western blot protein assay were made up of 11 CIN3 lesions, 10 normal samples from adjacent regions and 13 normal samples from women with no histologically detectable abnormality. They were resuspended in sample buffer containing 2.3% sodium dodecyl sulphate. 0.0625M Tris/HCL, pH7.9, 10% glycerol, 5% β-mercaptoethanol, and bromphenol blue. Samples were sonicated for 10 seconds and then boiled for 2min. The samples had equal protein content, as determined by the method of Bradford (9) and were split in two and run on two sides of the same SDS-polyacrylamide gel.

Following eletrophoresis, one half of the gel was stained with Coomassie blue and then destained, while the other half was transferred to nitrocellulouse and probed with antibody to Brn-3a or Brn-3b (Bab Co Ltd) as previously described (10). Levels of Brn-3a or Brn-3b in each sample were quantified by densitometric scanning of the resulting autoradiograph. Differences in the level of total protein in each sample were determined by scanning the actin band on the stained portion of the gel to normalise the samples (10).

Results

To measure the Brn-3a and Brn-3b mRNAs in the limited amounts of material available from human cervical biopsies, we used a reverse

transcriptase/polymerase chain reaction (RT/PCR) assay which we have previously used to measure the levels of Brn-3a/Brn-3b and other POU family transcription factors in limited amounts of material (6-7). The levels of amplification product obtained following the amplification of the Brn-3a and Brn-3b mRNAs with specific primers was compared to that of the constitutively expressed cyclophilin mRNA in each sample to control for any differences in the amount of total RNA or amplification efficiency in each sample (Figure 1).

Following densitometric scanning of results of this kind, the level of the Brn-3a and Brn-3b mRNAs was quantitated in sixteen cervical samples derived from individuals with no histologicaly detectable cervical abnormality and fourteen CIN3 (CINIII) samples. The levels of mRNA observed in replicate determinations using the same sample varied by less than 10% confirming the reliability of the assay (data not shown).

The results of the analysis (Figure 2 and Table 1) revealed virtually no change in the expression of Brn-3b mRNA between the normal and CIN3 samples with a similar mean level and an overlapping range of values between the two samples. In contrast the mean level of the Brn-3a mRNA was elevated approximately three hundred fold in the CIN3 samples compared to the normals (Figure 3 and Table 1). Indeed the CIN3 samples with the lowest level of Brn-3a mRNA still had approximately six fold more mRNA than the highest normal samples. The levels of Brn-3a mRNA were at the limit of detection in five of the normal samples and were undetectable in the remainder.

Table 1

Levels of Brn-3a and Brn-3b in individuals with no detectable cervical abnormality, CIN3 lesions and regions adjacent to a CIN3 lesion (N- CIN)

	Normal	CIN3	N-CIN
Brn-3a	0.001 +/-0.003	0.46+/-0.30	0.42+/-0.31
Brn-3b	0.1+/-0.11	0.11+/-0.18	0.07+/-0.09
Brn-3a/Brn-3b	0.01	4.2	6.0
Sample size	16	14	16

These dramatic changes in the level of Brn-3a mRNA in the CIN3 samples compared to the similar levels of Brn-3b resulted in a very large elevation in the Brn-3a/Brn-3b ratio in the CIN3 compared to normal samples (Table I). As the ratio between the Brn-3a activator and the Brn-3b repressor critically determines the activity of the HPV URR (11) it is likely that this effect plays a key role in the activation of HPV gene expression in the CIN3 patients.

Evidently, this elevation of Brn-3a could either be confined to the CIN3 region of these patients or represent a more widespread elevation in the patients with CIN3 which also occurred in adjacent histologicaly normal regions of the cervix. To distinguish these possibilities we determined the levels of Brn-3a and Brn-3b in further material from CIN3 patients which was derived from adjacent regions of the cervix with no detectable abnormality and which had no detectable expression of HPV E6 and E7 mRNA or HPV 16 DNA (data not shown). In these experiments, these samples exhibited a mean level of Brn-3a mRNA which was only marginally lower than that of the CIN3 region and dramatically more than was detectable in the normal samples obtained from individuals with no histologicaly detectable abnormality anywhere in the cervix (Figure 3 and Table 1).

Together with a similar level of Brn-3b expression in these samples (Figure 2) this resulted in a Brn-3a/Brn-3b ratio which was similar to that in the CIN3

samples and much greater than that in normal individuals (Table 1). Hence the elevated levels of Brn-3a and of the Brn-3a/Brn-3b ratio appear to be characteristic of both the CIN3 area and the adjacent area of the cervix of individuals with CIN3 compared to normal cervical tissue rather than of the actual area of malignancy alone.

To further extend these results, to the protein level, we carried out western blotting experiments using extracts from the various samples and antibodies to Brn-3a and Brn-3b. These results (figure 4 and 5) paralleled the results obtained at the mRNA level. Thus the mean level of Brn-3b was similar in all the groups (figure 4) whilst the level of Brn-3a was greatly elevated in CIN3 samples and normal samples from adjacent regions compared to the results obtained with normal samples from women with no detectable cervical abnormality (figure 5).

Although virtually all the samples from CIN3 patients had a uniformly high level of Brn-3a, we did identify a patient in whom four adjacent CIN3 sections had different levels of Brn-3a including one having the lowest level detected in a CIN3 sample (Table 2). This sample provided us with a unique opportunity to test the hypothesis that Brn-3a levels regulate the transcription of the HPV genome.

To do this, the level of HPV DNA and of HPV E6 and E7 RNA transcripts was measured using PCR amplification. In this experiment (Table 2) all four regions had a similar level of HPV DNA but the level of HPV E6 and E7 RNA transcripts detected was different in each case when compared to the level of the invariant cyclophilin mRNA. No signal was obtained with the HPV primers and DNA or mRNA prepared from an HPV negative human neuroblastoma cell line confirming the specificity of the assay. Most interestingly, HPV E6 and E7 RNA was undetectable in the sample with minimal Brn-3a levels, was present at low level in the sample with intermediate Brn-3a levels and was found at high levels in the two samples with high Brn-3a levels (Table 2). As expected the level of Brn-3b was similar in all the samples (data not shown).

Table 2

Levels of Brn-3a mRNA HPV DNA and HPV RNA in a single individual with CIN3

Section DNA level	Brn-3a mRNA level	HPV E6/E7 RNA level	HPV
1	0.075	undetectable	0.87
2	0.72	0.31	0.80
3	0.30	0.04	0.90
4	0.63	0.30	0.80

Values are in arbitary densitometric units equalised for the level of cyclophilin mRNA in each sample.

Discussion

The data presented here indicate that the level of the Brn-3a cellular transcription factor increases in patients with CIN3 lesions compared to the levels observed in normal cervical cells from individuals with no detectable cervical abnormality. The very dramatic increase in the levels of Brn-3a contrast with the

similar levels of Brn-3b in all the samples and result in a rise in the Brn-3a/Brn-3b ratio. Since Brn-3a can activate via the HPV URR whereas Brn-3b inhibits both its basal activity and its activation by Brn-3a (11), changes in this ratio are likely to play a critical role in regulating URR activity.

The elevated level of Brn-3a in the CIN3 samples compared to that in normal cells suggests a role for this elevation in the activation of HPV gene expression and the resulting cellular changes. On the basis of this initial comparison however, it remained possible that the elevated level of Brn-3a was simply a consequence of the altered nature of these cells. Thus in this case, the elevation would reflect some property of the cells resulting from their transformation by HPV rather than being involved in the actual activation of HPV gene expression.

However, elevated expression of Brn-3a in histologicaly normal segments of the cervix adjacent to the CIN3 region was also observed compared to the level observed in normal samples from individuals with no detectable cercival abnormality. These samples did not exhibit any detectable HPV DNA or RNA in accordance with previous results (3). Hence elevation of Brn-3a appears not to be characteristic solely of the pre-malignant cells in these individuals. This may indicate that the region adjacent to the CIN3 lesion, although histologicaly normal, is also abnormal in that it over-expresses Brn-3a. Alternatively our findings may reflect a widespread elevation of Brn-3a in the cervix of individuals with CIN3 either due to exposure to a specific stimulus, or to differences within the human population in cervical Brn-3a levels.

In either case, it is clear that a dramatic elevation of Brn-3a levels occurs in the cervical cells of some individuals either throughout the cervix or in a relatively restricted region. In those individuals of this type also infected with HPV-16 and HPV-18, such an elevation will result in activation of the URR leading to E6 and E7 expression and cellular alterations in the cervical transformation zone at the junction of the endocervix and ectocervix where cervical tumours appear.

In agreement with this idea, the level of HPV transcripts in different CIN3 samples from a single individual directly correlated with the level of Brn-3a in each sample. Hence the elevation of Brn-3a levels could play a critical role in the

activation of viral transcription and disease progression, although other factors such as viral type, viral load and differences in cellular susceptibility to transformation would be responsible for the precise localization of the malignant lesions.

These considerations focus attention on the stimuli which regulate Brn-3a expression in cervical cells. Stimuli which can regulate the levels of Brn-3a and Brn-3b in opposite directions in neuronal cells have previously been defined (12, 5, 13). Alternatively, it is possible that the elevated Brn-3a levels in individuals with CIN3 reflect a variation in the human population produced during cervical development and differentiation which affects HPV transcription. Whatever the case, it is clear that a dramatic elevation in Brn-3a levels is characteristic of women with high grade cervical lesions compared to individuals with no detectable cervical abnormality.

Example 2

Materials and Methods

Plasmid constructs

The expression vector pLTRpoly(ATCC) containing the full length of the class IV POU domain transcription factors Brn-3a and Brn-3b under the Moloney murine leukaemia virus promoter (MoMuLV) have previously been described (14). The antisense Brn-3a construct was cloned within the pJ5 vector polylinker, under the control of the glucocorticoid-inducible mouse mammary tumour virus promoter (15).

Stable transfection and isolation of clonal cell lines

SiHa (ATCC) and C33 (ATCC) cell lines were grown in Minimum essential medium (Eagle) with Earle's BSS, supplemented with 10% fetal bovine serum, 0.1mM non-essential amino acids and 1.0mM sodium pyruvate. The Brn-3 expression vectors were cotransfected with pCi-neo (Promega) neomycin resistant vector into both cell lines by calcium phosphate-mediated transfection method (16).

Typically, 15μg of the respective recombinants plus 3μg of the neomycin resistant plasmid were co-transfected into 80% subconfluent SiHa and C33 cells in 10cm plates, and media were supplemented subsequently with g418 (Gibco) to a

final concentration of 800µg/ml. Putative clones began emerging after about 10 days and were subsequently isolated with cloning rings and cultured in medium supplemented with 800µg/ml G418. Antisense and control clones were treated with 1µM dexamethasone 24 hours prior to protein extraction.

Western blotting

Harvested cells were resuspended in 100ul extraction buffer (20mM Hepes (pH7.8), 450mM NaCl, 0.4mM EDTA, 0.5mM DTT, 25% glycerol 0.05mM phenylmethylsulphonyl fluoride (PMSF)) and freeze-thawed. The protein concentration of the supernatant was determined and thus used for SDS-page electrolysis as earlier reported (1), though with some modification for HPV-E6 protein analysis with shorter SDS-PAGE resolution time.

The gel was blotted onto membrane (Amersham) and the membrane was blocked for 2 hours with 10% Marvel (fat-free milk) and incubated with 1:500 HPV-16-E6 antibody (Santa Cruz) for 16 hours overnight at 4°C, washed 5 times with 0.1% Tween 20, then incubated with HRP conjugated mouse secondary antibody (Santa Cruz) for an hour.

Analysis of cellular growth rate and saturation density

To analyse the growth rate of both the parental and clonal cells, a basic method of counting the amount of viable cells in a haemacytometer's chamber using a light microscope was employed. Routinely, each putative clone or control cell was seeded with an initial density of $1x10^4$ cells in three groups of eight, at regular intervals of between 8, 16 and 24 hours, subsequent groups of three were trypsinized, washed, resuspended in appropriate percentage of trypan blue medium and then counted. For determination of the saturation density, the same method was used except for the longer time interval of about 3 days, during which the cells were allowed to proliferate freely without passage.

Anchorage independent growth

Determination of anchorage independent proliferation was established by

growing cells in soft agar. 3ml of 10^3 clonal or parental cells resuspended in low melting point agarose (Gibco) dissolved in G418 supplemented media with or without dexamethasone to a final constitution of 0.33% was overlaid in triplicate 60mm plates containing 0.5% low melting point agarose dissolved in appropriate medium. The plates were immediately incubated at 37°C and colonies scored after 10 days.

Results

To investigate the effect of manipulating the expression of Brn-3a in cervical cancer cell lines, we utilised the SiHa cell line which contains a single integrated HPV-16 genome and, for comparison, the C33 transformed cervical cell line which does not contain any HPV DNA (17-19). To over-express Brn-3a, the cells were transfected with an expression vector in which expression of Brn-3a is driven by the moloney murine leukaemia virus promoter (MoMuLV) which we have previously used to successfully over express Brn-3a in neuronal cells (14). A similar vector was also used to over-express Brn-3b in these cells for comparison.

Similarly, to reduce the level of endogenous Brn-3a we used a construct in which an antisense transcript of Brn-3a is produced under the control of the glucocorticoid-inducible mouse mammary tumour virus promoter (MMTV) which we have similarly previously used to reduce Brn-3a levels in neuronal cells (15). Similar transfections were also carried out using the expression vectors lacking any insert in order to produce control cell lines. In each case, stably transfected cell lines were selected on the basis of their neomycin resistance which was encoded on the plasmid vector.

Clonal cell lines isolated in this way were first tested to determine whether they contained altered levels of Brn-3a as determined by Western blotting with a specific antibody. In these experiments (Figure 6) clear over expression of Brn-3a was observed in several cell lines transfected with the Brn-3a expression vector compared either to parental untransfected cells, cells transfected with the expression vector alone or cells transfected with the Brn-3b expression vector.

In contrast, several cell lines obtained by transfection with the Brn-3a

antisense construct showed only minimal reduction of Brn-3a levels in the absence of dexamethasone to induce the MMTV promoter. However, a clear reduction in Brn-3a levels was observed in several of these cell lines when the cells were treated with dexamethasone resulting in the induction of the antisense construct. This effect was observed in both the SiHa cells and in the C-33 cells transfected with the Brn-3a antisense construct (Figure 6). In contrast, no effect of dexamethasone on endogenous Brn-3a levels was observed in either of the parental cell lines when treated with dexamethasone or in the cell lines transfected with expression vector lacking any insert (data not shown). Similarly, no alteration in exogenous Brn-3a levels was observed in the cell lines obtained by tranfection with the Brn-3a sense construct under the control of the MoMuLV promoter. These data thus indicate that the cell lines engineered to over-express Brn-3a do indeed show a specific elevation of Brn-3a levels whereas the antisense cell lines show decreased expression of Brn-3a particularly when grown in the presence of dexamethasone to induce the antisense construct. The cell lines showing respectively the greatest elevations or reductions in Brn-3a levels were selected for further study. Similarly, the SiHa and C33 cell lines over engineered to express Brn-3b showed a specific elevation of Brn-3b levels which was not observed in the other cell lines (data not shown).

To determine whether these alterations in Brn-3a and Brn-3b levels did produce a change in the level of HPV gene expression, the cellular extracts were also western blotted with antibody to the HPV E6 protein. In these experiments (Figure 7) the SiHa cells engineered to over express Brn-3a showed a small increase in HPV expression over the control parental SiHa cells or SiHa cells containing only plasmid vector whilst the cells engineered to overexpress Brn-3b showed a similar small decrease in HPV gene expression. Significantly however, the two cell lines expressing the antisense construct showed a clear decrease in the level of E6 gene expression which was greatest in cell line 5 paralleling the greater reduction in Brn-3a levels in this cell line compared to the antisense cell line 3. As expected, no HPV gene expression was detected in any of the cell lines derived from C-33 cells which are not transformed with HPV.

These data indicate therefore that the HPV gene expression which occurs in

the SiHa cell line appears to be dependent upon the expression of Brn-3a in these cells since it can be specifically reduced by decreasing Brn-3a levels using an antisense approach. We therefore wished to establish whether such alteration in HPV gene expression mediated via Brn-3a resulted in alterations in the growth rate of the manipulated cells. Evidently, the C33 cells serve as an important control for these experiments since any direct effect of manipulating Brn-3a levels on cellular growth would also be observed in these cells whereas this would not be the case if the effect in SiHa cells is mediated via the alteration in HPV gene expression which would not occur in the C33 cells.

We therefore measured the growth rate of the various different clones over a 72 hour period. In the experiments with the SiHa clones, the parental SiHa cells and the cells transfected with empty expression vector alone showed a similar growth rate (Figure 8a) indicating that the selection of stably transfected cell lines does not produce cell lines with enhanced growth rates. Interestingly, over expression of Brn-3a resulted in a somewhat enhanced growth rate of the SiHa cells whereas over expression of Brn-3b produced a correspondingly reduced growth rate (Figure 8a).

Most importantly, although the cells engineered with antisense Brn-3a showed a similar growth rate to parental cells in the absence of dexamethasone, their growth rate was dramatically reduced by treatment by dexamethasone, although this treatment had no effect on the growth of parental cells (Figure 8b).

These experiments thus indicate that the reduced Brn-3a expression in the antisense SiHa cells is paralleled not only by reduced HPV gene expression but also by reduced growth rate. In similar experiments in the HPV negative C-33 cells (Figure 9) all the cell lines showed similar growth rates. Hence manipulating the expression of Brn-3a or Brn-3b in a cervical cell line which does not express HPV, does not result in altered growth rates.

As well as measuring the effect of manipulating Brn-3a expression on cellular growth rate, we also wished to determine whether such manipulation would affect the saturation density of the cells since the loss of contact inhibition resulting in growth to higher densities is characteristic of cancer cells.

The various cell lines were therefore plated out and grown over a period of

several days in order to determine their saturation density. In these experiments (Figure 10) similar saturation densities were observed in the parental SiHa cells, the cells transfected with plasmid expression vector alone and the cells overexpressing Brn-3a. However, a clearly reduced saturation density was observed in the SiHa cells over expressing Brn-3b (Figure 10a).

Similarly, two distinct SiHa cell lines containing the transfected antisense contruct showed a clear reduction in saturation density compared to the parental cells (Figure 10b). This reduction was greater in cell line 5 compared to cell line 3 paralleling the greater reduction in HPV gene expression in this cell line (see Figure 7).

Moreover, the saturation density of both the antisense cell lines was further reduced by full induction of antisense expression using dexamethasone whereas no effect on saturation density was observed when the parental cells were treated in this way confirming that this effect was specific to the cells containing the antisense construct (Figure 10b). As in the cell growth experiments, all the C-33 derived cell clones showed similar saturation densities which were unaffected by dexamethasone (Figure 11) indicating that the effects in SiHa cells correlate with the effect of Brn-3a on HPV gene expression.

Having established the effect of manipulating Brn-3a expression on the growth and saturation density of the cell lines when grown attached to culture dishes, we wished to determine the effect of such manipulation on their ability to grow in an anchorage independent manner since this is an important feature of tumour cells necessary for their growth *in vivo*. We therefore measured the ability of the various cell lines to form colonies in soft agar. As indicated in Figure 12a, the SiHa cells showed a clear ability to form colonies in soft agar as expected and this was not affected in the cells containing the plasmid expresion vector or in the cells over-expressing Brn-3a.

However, a reduced rate of colony formation in soft agar was observed in the cells over expressing Brn-3b paralleling their reduced growth rate and saturation density when grown attached to culture dishes. Moreover, a still greater reduction in colony formation of approximately four fold was observed in the two different cell

lines containing the antisense Brn-3a contruct (Figure 12a). This colony formation was reduced even further upon treatment of the antisense cells with dexamethasone with colony formation being virtually undetectable in cell line 5 paralleling its greater reduction in HPV gene expression and saturation density. This effect of dexamethasone was specific to the antisense cell lines since no effect of dexamethasone on colony formation was observed in the parental cells.

To determine whether these effects of manipulating Brn-3a levels on anchorage independent growth were dependent upon the altered level of HPV gene expression, we carried out similar experiments in the C-33-derived cell lines. As illustrated in Figure 12b however, all the various cell lines showed a similar ability to form colonies in soft agar which was not in any way affected by the alteration of Brn-3a or Brn-3b levels. Hence, the effects on anchorage independent growth observed in the SiHa cell lines are correlated with the effect of Brn-3a on HPV gene expression in the same manner as the effect on the growth of cells attached to culture dishes.

Discussion

We have demonstrated for the first time that the manipulation of Brn-3a expression can affect the levels of gene expression from an integrated HPV genome in a transformed cervical cell line. Thus, SiHa cell lines transfected with an antisense Brn-3a expression plasmid showed a clearly reduced expression of HPV. Hence, the expression of the single endogenous HPV genome in SiHa cells appears to depend upon the expression of Brn-3a in these cells such that when Brn-3a expression is reduced, HPV gene expression is correspondingly reduced. This effect evidently parallels our previous finding that a motif in the HPV URR can be transactivated by Brn-3a in co-transfection assays involving promoter-reporter constructs (20) and extends it to an endogenous HPV genome. Interestingly, despite the increased Brn-3a levels observed in cells transfected with an expression vector for Brn-3a, the levels of HPV gene expression were only slightly increased suggesting that HPV gene expression is already maximally stimulated in SiHa cells by the significant level of endogenous Brn-3a in these cells. Interestingly however, HPV gene expression

could be reduced by over-expressing Brn-3b in SiHa cells paralleling the ability of Brn-3b to repress URR activity in co-transfection experiments (20). These experiments thus establish Brn-3a expression as being critical for the maintenance of HPV gene transcription in a cervical cancer cell line. We also demonstrated that the growth characteristics of such a cell line are similarly dependent upon Brn-3a. Thus, the inhibition of Brn-3a expression using an antisense approach led to reduced cellular growth rate, saturation density and the ability to grow in an anchorage independent manner. Several lines of evidence indicate that this effect is dependent upon the ability of Brn-3a to modulate HPV gene expression rather than to a direct effect of Brn-3a on the cell. Thus, no effect of reduced Brn-3a levels on cellular growth, saturation density or anchorage independence was observed in the C-33 cells which showed a similar reduction in Brn-3a levels but which are not transformed with HPV. Similarly, over expression of Brn-3b in the SiHa cells which also reduced HPV gene expression also resulted in reduced growth rate, saturation density and anchorage independent growth, although the effects were not as dramatic as reducing Brn-3a levels paralleling the less dramatic effect of over-expressing Brn-3b on HPV gene expression. Lastly, it should be noted that in the C4-1 cervical carcinoma cell line, reduction of E6 and E7 expression with an antisense approach, similarly resulted in reduced cellular proliferation (21).

Thus, simply by manipulating Brn-3a levels it is possible to alter HPV gene expression and thereby alter the growth characteristics of the tumour cells in terms not only of growth rate, and independence from contact inhibition but most importantly in terms of anchorage independence which is a key requirement for tumourigenesis *in vivo*. This association of Brn-3a with HPV gene expression and the characteristics of transformed cervical cells is of particular interest in view of our previous finding that Brn-3a is over-expressed in the transformation zone of women with CIN3 compared to women with no detectable cervical abnormality (1). Such over-expression of Brn-3a is likely therefore, in view of our current results, to play a key role in the elevated HPV gene expression observed in the transformed cells which is critical for oncogenic transformation.

Hence the elevated levels of Brn-3a observed in CIN3 material and in

cervical cancer cell lines appear to play a key role in the elevated expression of HPV and thereby in determining the transformed phenotype. These considerations evidently focus attention on the manner in which Brn-3a expression becomes elevated in women with CIN3. In our previous study (1) we were able to show that similar elevated expression of Brn-3a occurs in histologicaly normal segments of the cervix adjacent to the CIN3 region which do not contain detectable HPV DNA or RNA and we have now extended these findings to show that Brn-3a expression is elevated throughout the cervix in women with CIN3 (DN, unpublished data). This widespread elevation in Brn-3a levels in women with CIN3 may be dependent upon their exposure to an environmental factor which raises Brn-3a levels or alternatively could reflect a genetic difference in the Brn-3a gene regulatory region which results in elevated expression of Brn-3a in these women. In this latter case, this genetic polymorphism would represent a risk factor for cervical cancer similar to having a p53 gene encoding a protein with an arginine at position 72 resulting in enhanced degradation by the HPV E6 protein (22).

Whatever the cause of the elevated level of Brn-3a however, it is clear that in individuals having such elevation, infection with HPV-16 or HPV-18 will result in the activation of the HPV URR leading to E6 and E7 expression and cellular alterations in the cervical transformation zone at the junction of the endocervix and the ectocervix where cervical tumours appear. Hence the elevated levels of Brn-3a, whether caused by environmental or genetic causes, would play a critical role in activation of viral transcription and disease progression., although other factors such as viral type, viral distribution and cellular susceptibility to transformation would be responsible for the precise localization of the malignant lesions.

Most importantly, the fact that the level of HPV gene expression and the abnormal growth characteristics of cervical cancer cells can be reversed by reduction of Brn-3a expression, makes this factor an attractive target for therapeutic intervention. This could involve the reduction of endogenous Brn-3a expression either by pharmacological manipulations to reduce that activity of the Brn-3a gene promoter or by the use of gene delivery vectors to deliver Brn-3a antisense constructs similar to those utilised here.

To investigate further the potential utility of such forms of therapy, we are currently investigating whether the SiHa cells with reduced Brn-3a levels show reduced tumorigenicity when grown in nude mice and also whether the growth rate of an established tumour can be manipulated *in vivo* by using viral vectors expressing the antisense strand of the Brn-3a gene. It is already clear however from the experiments described here that the Brn-3a factor which is over expressed in women with CIN3, plays a key role in HPV gene transcription and thereby regulates the growth characteristics of cervical carcinoma cells.

Example 3

Nude mice were injected with SiHa cells stably transformed with the empty expression vector or with SiHa cells stably transformed with the Brn-3a anti-sense construct (see Example 2 above for details of these constructs). The mean size of tumours were assessed at regular intervals post injection. Figure 13 shows the results. In mice injected with cells stably transformed with the empty expression vector (Gp1), limited tumor growth was seen up to 30 days post injection. However, after 30 days tumors grew considerably. In mice injected with cells stably transformed with the Brn-3a anti-sense construct (Gp2), no or very little tumor growth was observed over the duration of the experiment. These experiments suggest that by reducing Brn-3a levels in cervical carcinoma cells the growth of those cells *in vivo* can be greatly reduced or even eliminated.

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CLAIMS

1. A product that binds, causes a decrease in intracellular levels of, or inhibits the activity of Brn-3a for use in the treatment, prevention or diagnosis of a cervical cancer attributable to HPV.

2. A produce according to claim 1 that inhibits the expression of Brn-3a.

3. A product according to claim 1 or 2 which comprises a polynucleotid capable of hybridising to Brn-3a mRNA.

4. A method of identifying a product that binds to Brn-3a comprising contacting Brn-3a, or a mimic of Brn-3a that can bind to a Brn-3a specific antibody, with a candidate substance and determining whether the candidate substance binds Brn-3a or the mimic of Brn-3a.

5. A method of identifying a product that causes a decrease in intracellular levels of Brn-3a comprising contacting a candidate substance with a cell or cell extract and determining whether the candidate causes a decrease in intracellular levels of Brn-3a.

6. A method of identifying a product that inhibits expression of Brn-3a comprising contacting a candidate substance with one or more components of the intracellular expression pathway of Brn-3a, or functional analogues of these components, and determining whether

(i) the candidate substance binds or inhibits component(s) that promote the expression of Brn-3a; or

(ii) the candidate substance stimulates component(s) that inhibit the expression of Brn-3a.

7. A method according to claim 6(i) wherein the component is a Brn-3a promoter, a Brn-3a promoter specific transcription factor, Brn-3a mRNA, or an intracellular protein that binds Brn-3a protein.

8. A method according to claim 6 comprising

(i) providing a test construct comprising a polynucleotide sequence with Brn-3a promoter activity operably linked to a coding sequence;

(ii) contacting a candidate substance with the test construct

under conditions that would permit the polypeptide encoded by the coding sequence to be expressed in the absence of the substance; and

(iii) determining whether the substance inhibits expression from the construct.

9. A method of identifying a product that inhibits the activity of Brn-3a comprising:

(i) contacting Brn-3a, or a mimic of Brn-3a that can bind to a Brn-3a specific antibody, with a candidate substance and determining whether the candidate substance binds Brn-3a or the mimic of Brn-3a; or

(ii) contacting an agent that can bind to Brn-3a to a candidate substance and determining whether the candidate substance binds to the agent; or

(iii)(a) contacting Brn-3a, or a mimic of Brn-3a with Brn-3a activity, with a candidate substance under conditions that would permit activity of Brn-3a, and (b) determining whether the candidate substance inhibits the activity of Brn-3a or the mimic of Brn-3a.

10. A method according to claim 9 (ii) wherein the agent is a component of HPV.

11. A method according to claim 9 (iii) wherein (a) the candidate substance is provided to Brn-3a, or the mimic of Brn-3a, and a Brn-3a responsive HPV component which is able to promote expression of HPV mRNA or proteins, or a functional analogue thereof, under conditions in which the Brn-3a responsive HPV component is active, and (b) determining whether the candidate substance inhibits the activity of the Brn-3a responsive HPV component.

12. A method of diagnosing cervical cancer in a female or identifying a female who is susceptible to cervical cancer comprising, determining whether cervical cells taken from a female express, or are predisposed to expressing, elevated levels of (i) Brn-3a, or (ii) Brn-3a mRNA.

13. A product identified by a method according to any one of claims 4 to 11.

14. A pharmaceutical composition comprising a product as defined in any one of claims 1 to 3 or 13 and a pharmaceutically acceptable carrier or diluent.

15. A method of treating a female suffering from cervical cancer comprising administering to the female a therapeutically effective amount of product as defined in any one of claims 1 to 3 or 13.

16. Use of a product that binds, causes a decrease in intracellular levels of, or inhibits the activity of Brn-3a for use in the manufacture of a medicament for the treatment, prevention or diagnosis of a cervical cancer attributable to HPV.

Fig.1A.

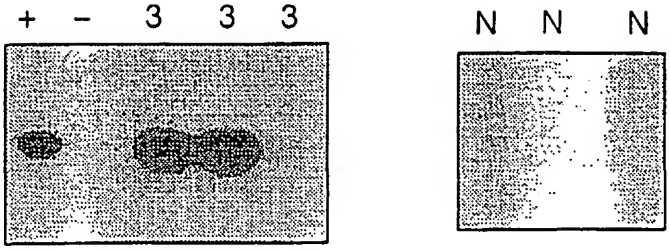


Fig.1B.

Fig.1C.

Fig.3.

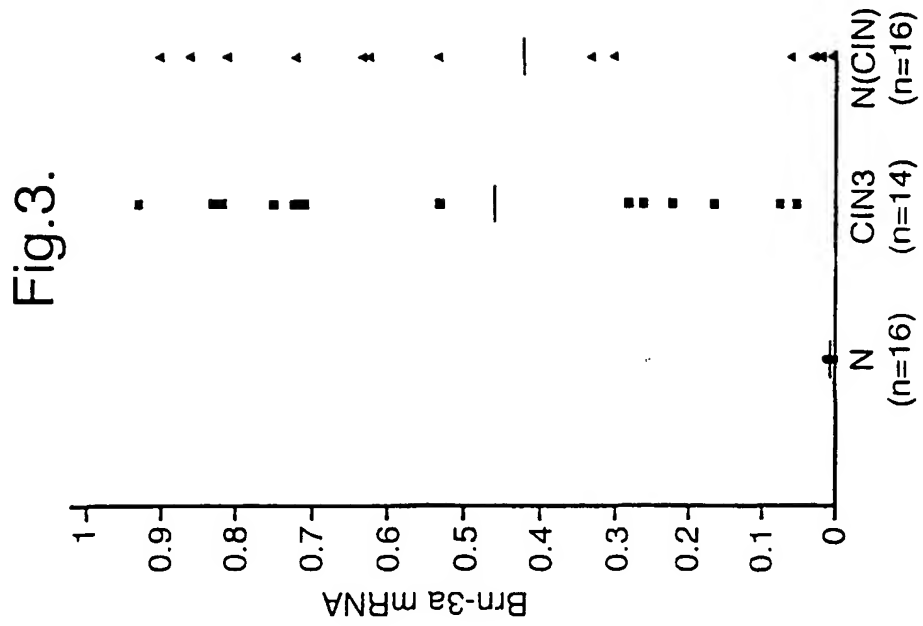

Brn-3a mRNA
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
N (n=16)
CIN3 (n=14)
N(CIN) (n=16)

Fig.2.

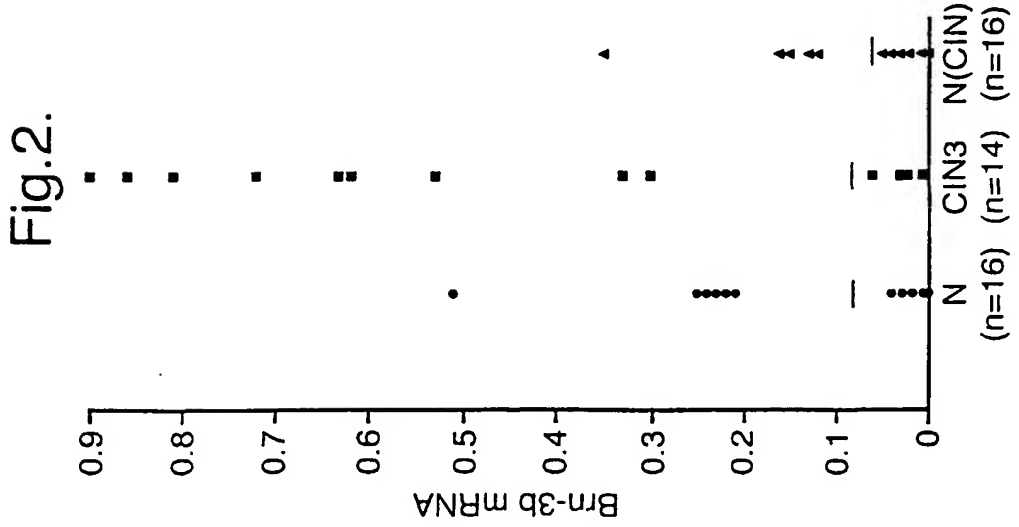

Brn-3b mRNA
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
N (n=16)
CIN3 (n=14)
N(CIN) (n=16)

Fig.5.

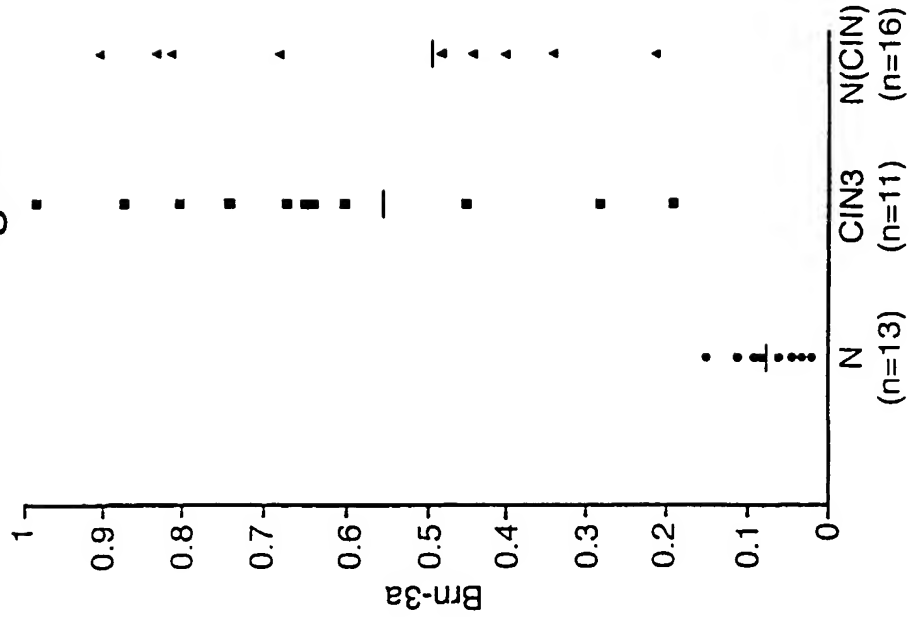

Brn-3a
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N (n=13)
CIN3 (n=11)
N(CIN) (n=16)

Fig.4.

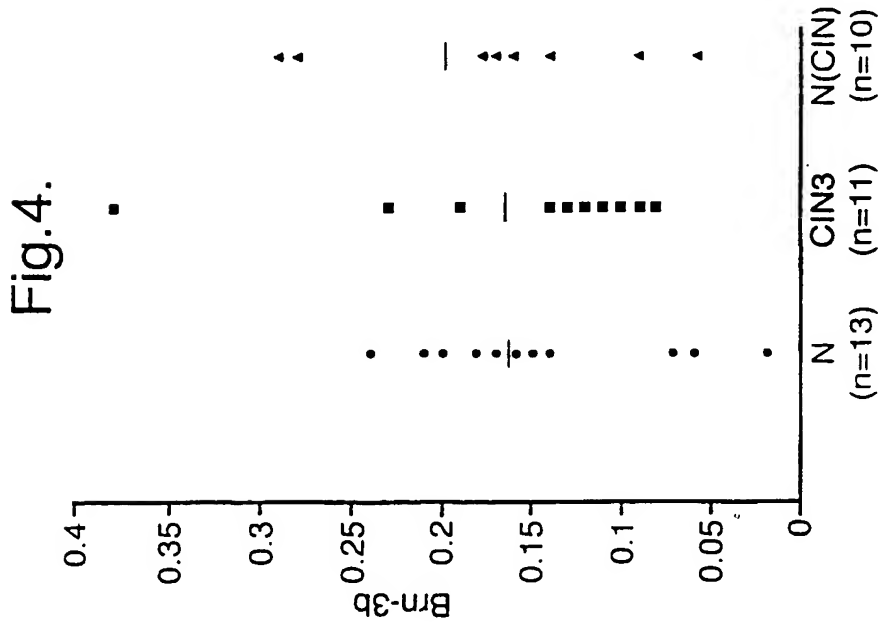

Brn-3b
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
N (n=13)
CIN3 (n=11)
N(CIN) (n=10)

Fig.6a.

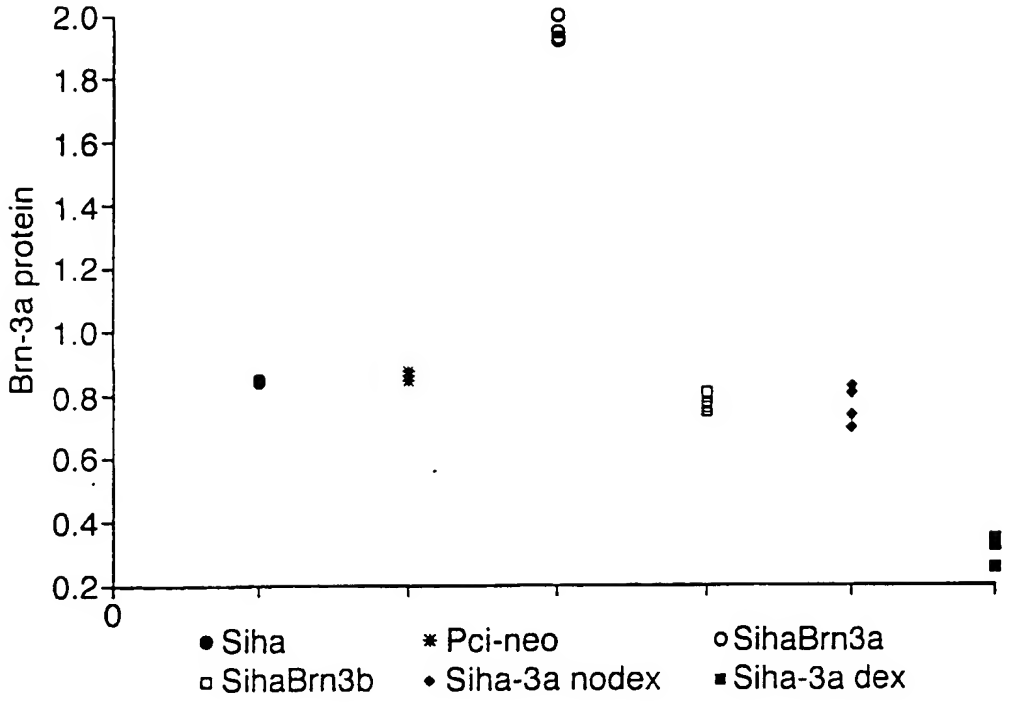

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Brn-3a protein
Siha
Pci-neo
SihaBrn3a
SihaBrn3b
Siha-3a nodex
Siha-3a dex

Fig.6b.

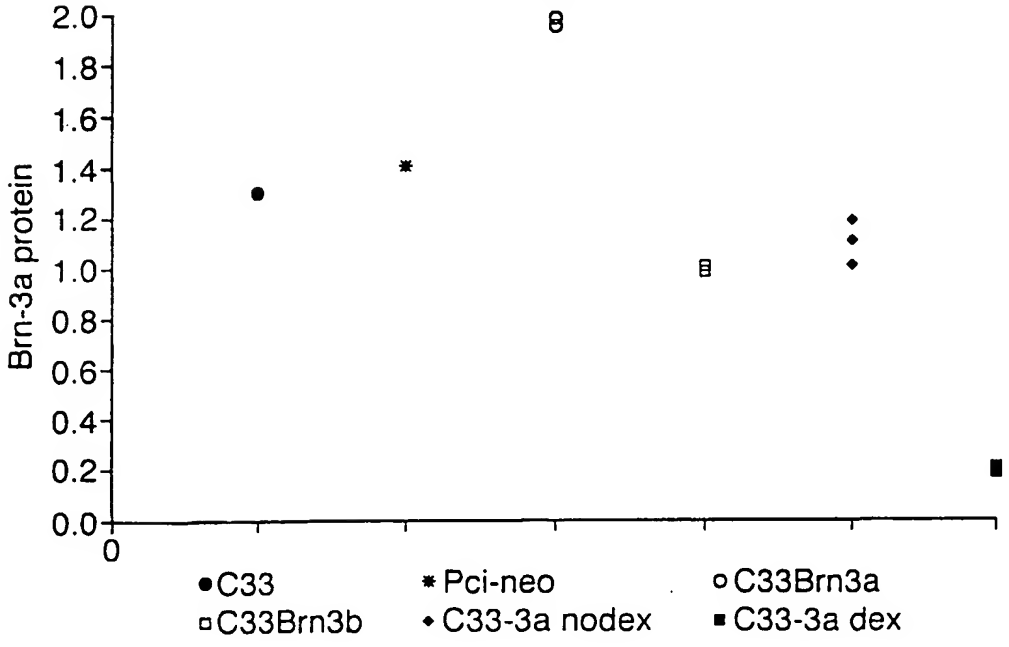

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
Brn-3a protein
C33
Pci-neo
C33Brn3a
C33Brn3b
C33-3a nodex
C33-3a dex

Fig.7.

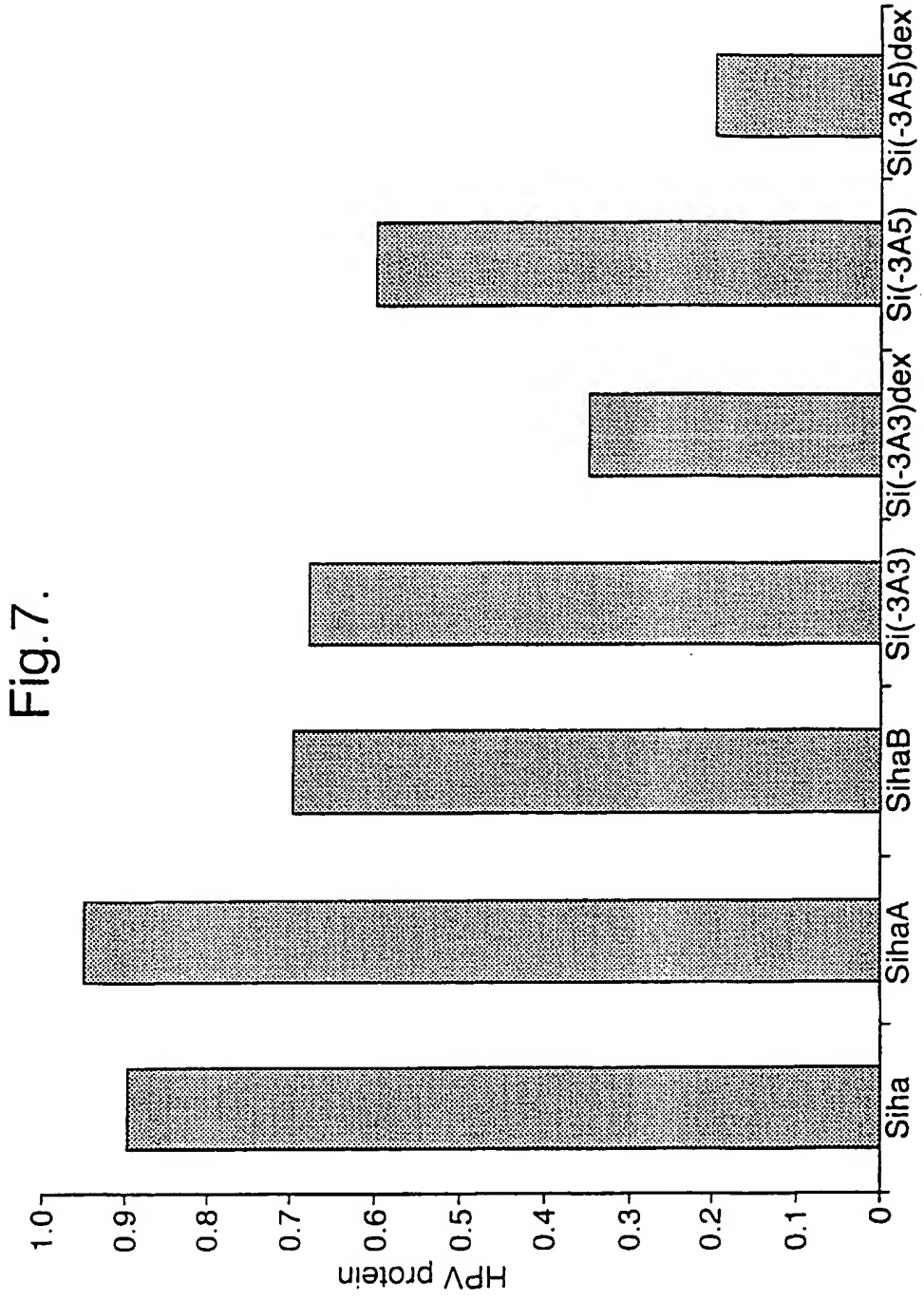
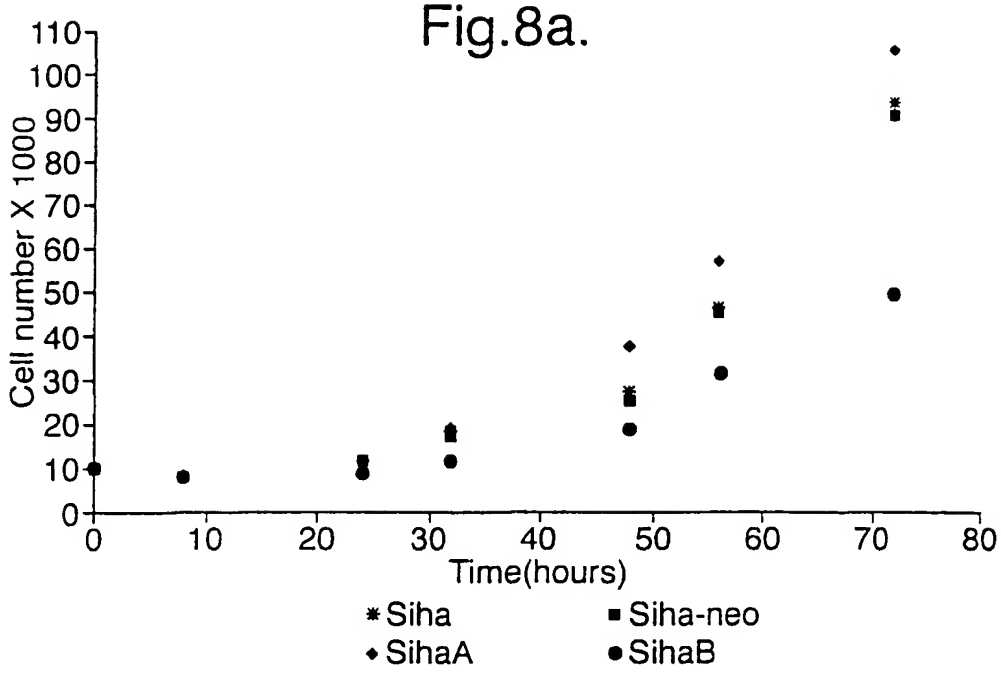

HPV protein
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Siha
SihaA
SihaB
Si(-3A3)
Si(-3A3)dex
Si(-3A5)
Si(-3A5)dex

Fig.8a.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0
10
20
30
40
50
60
70
80
90
100
110
0
10
20
30
40
50
60
70
80
Time(hours)
Siha
Siha-neo
SihaA
SihaB

Fig.8b.

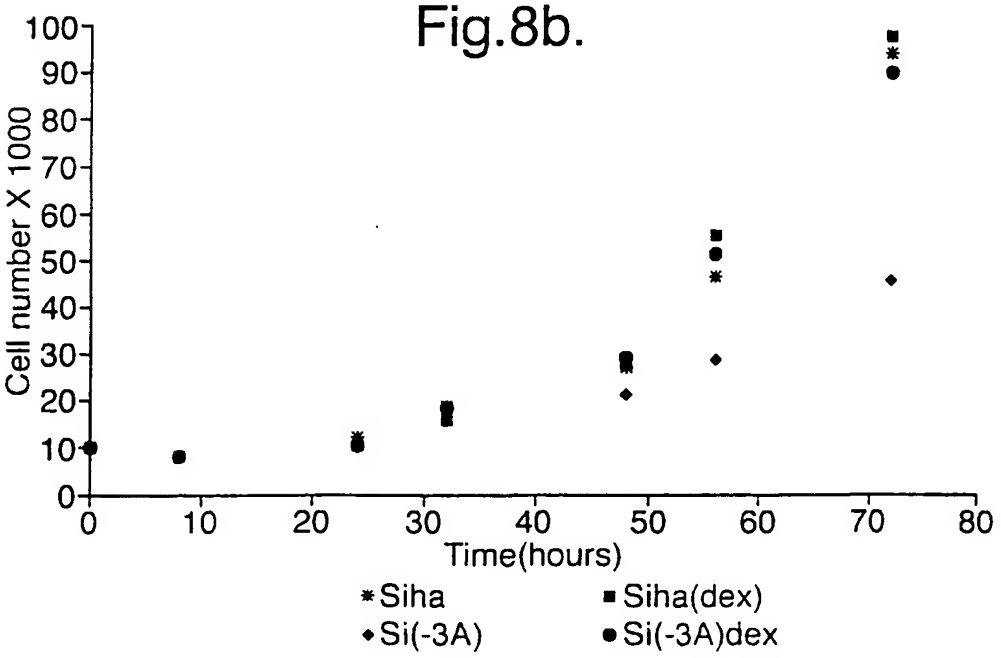
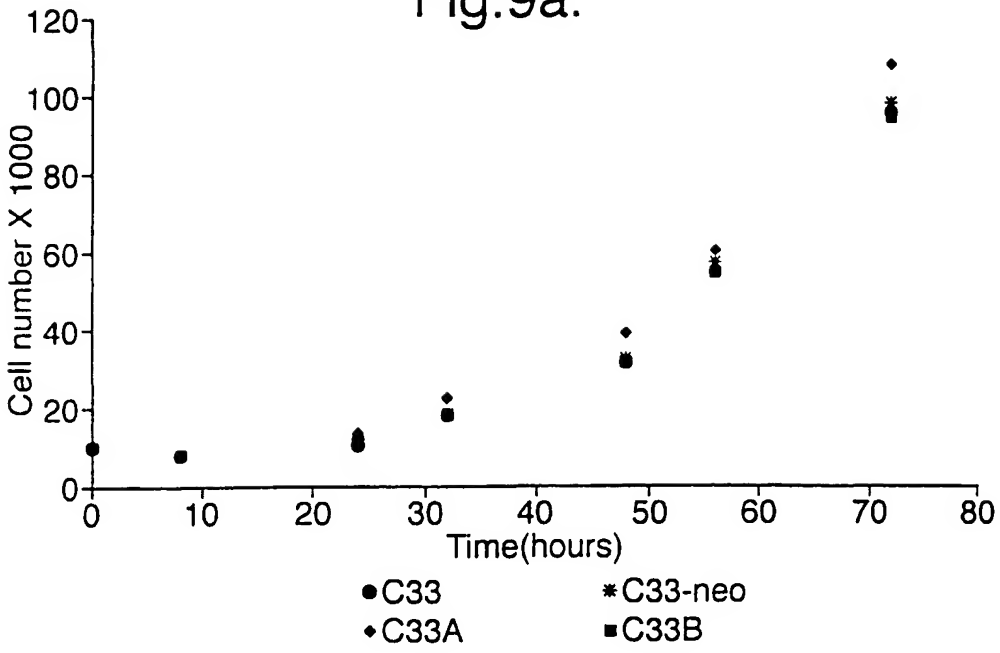

Cell number X 1000
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
Time(hours)
Siha
Siha(dex)
Si(-3A)
Si(-3A)dex

Fig.9a.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120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time(hours)
C33
C33-neo
C33A
C33B

Fig.9b.

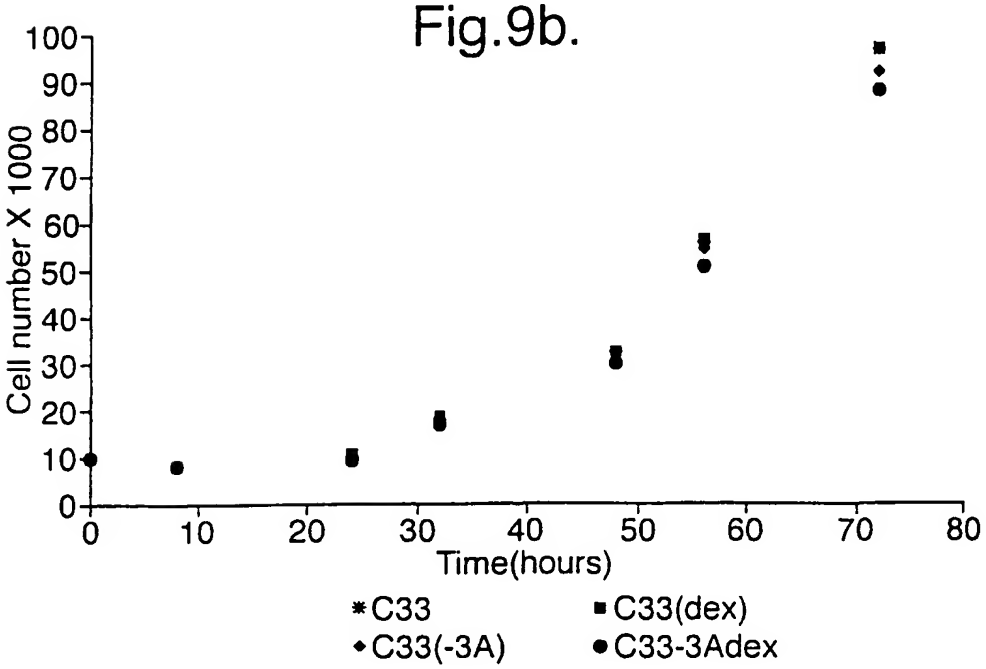

Cell number X 1000
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Time(hours)
C33
C33(dex)
C33(-3A)
C33-3Adex

Fig.10a.

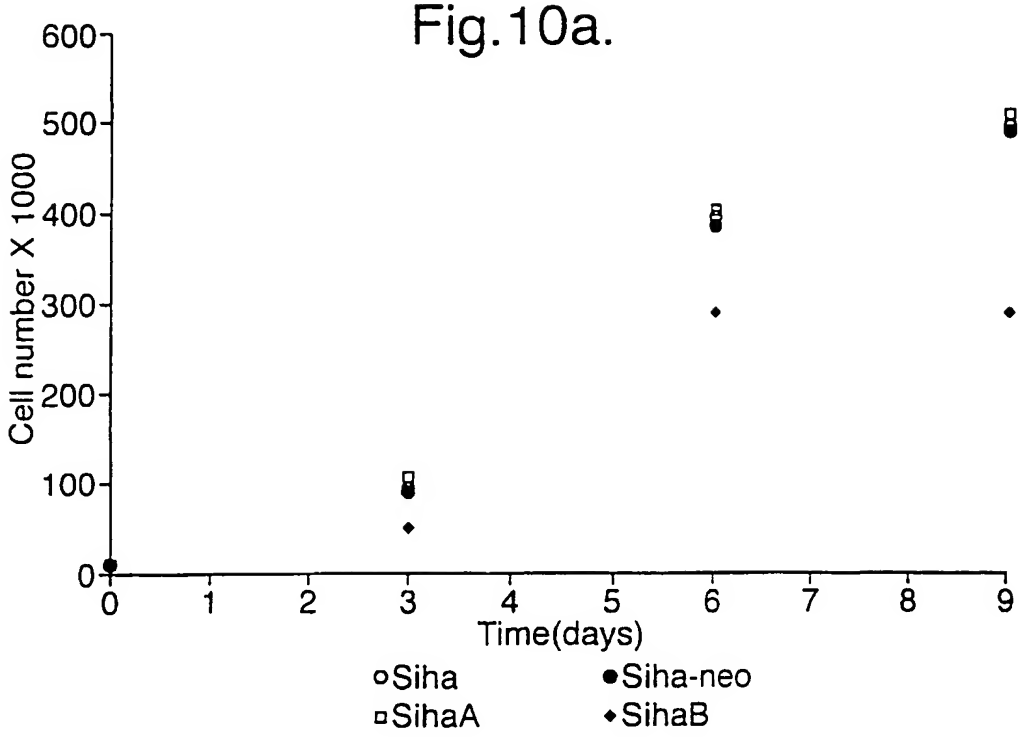


Fig.10b.

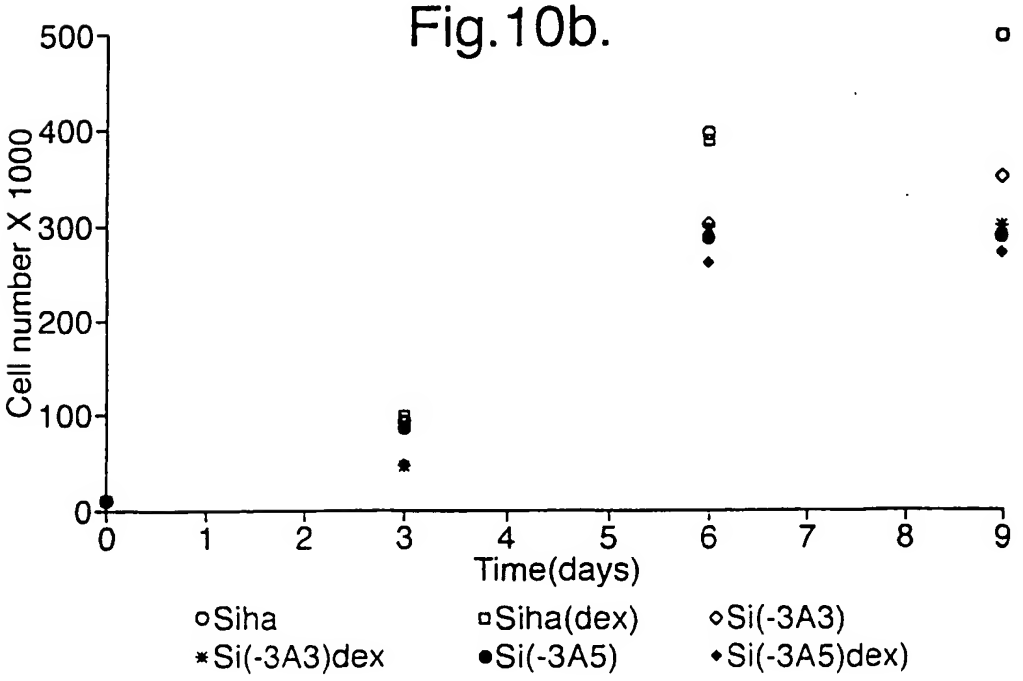


Fig.11a.

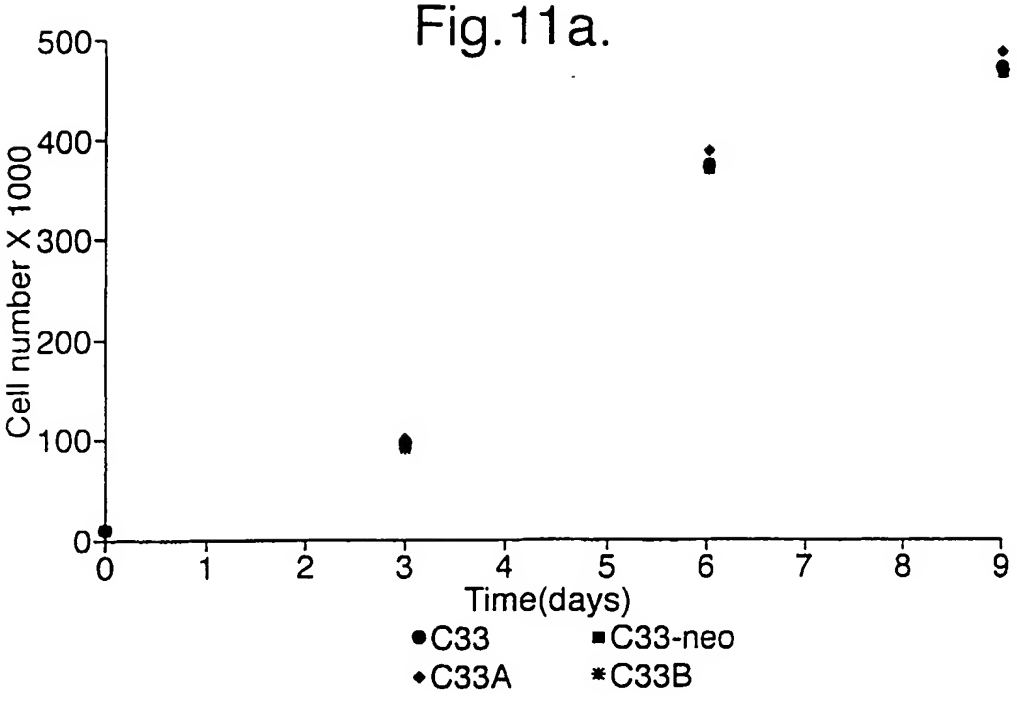


Fig.11b.

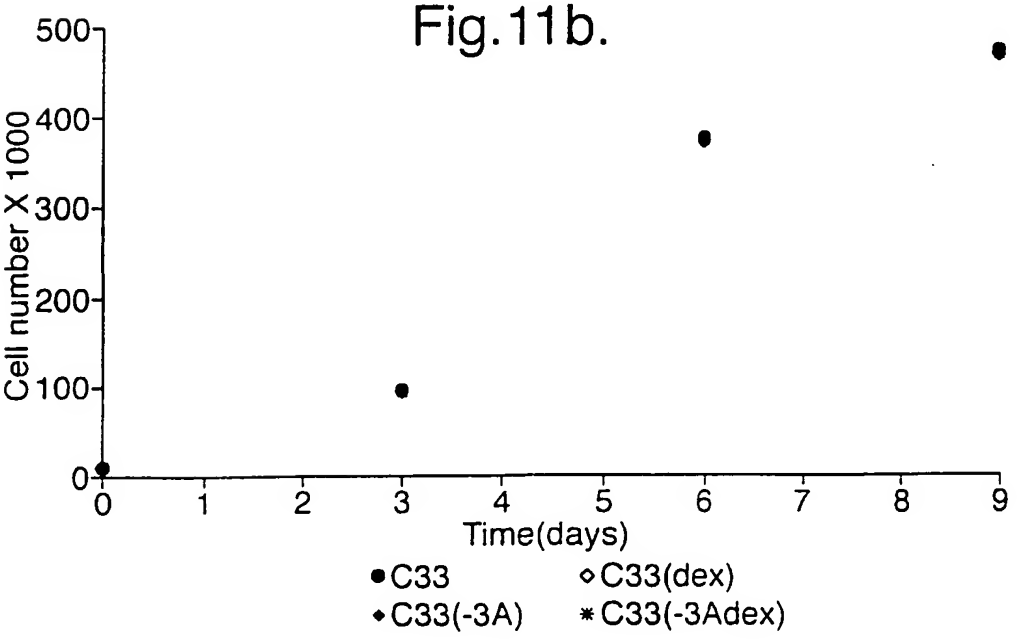


Fig.12a.

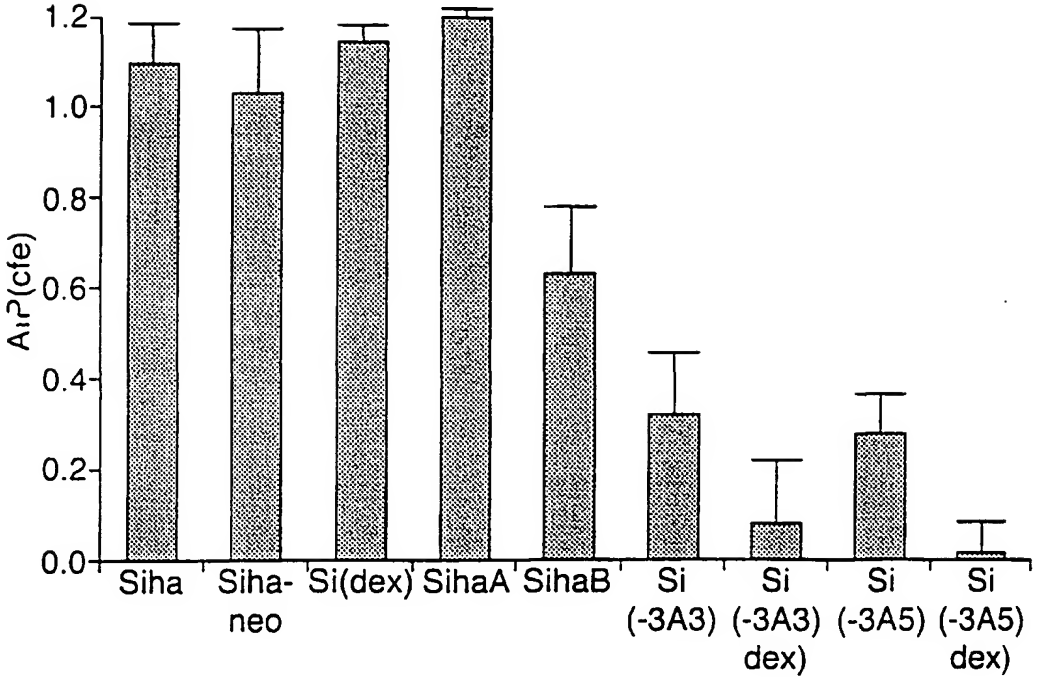


Fig.12b.

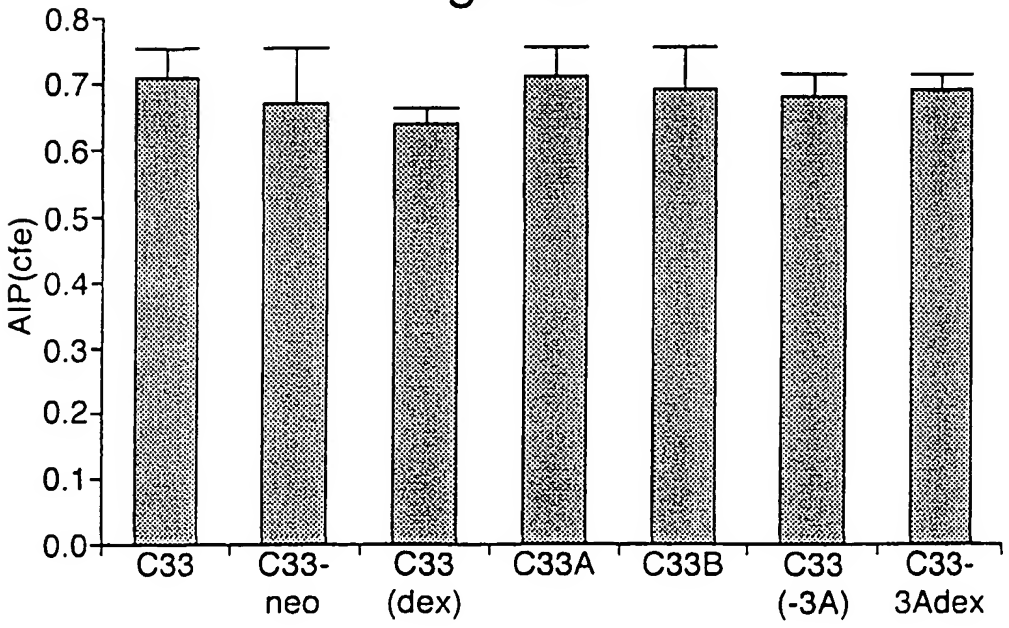
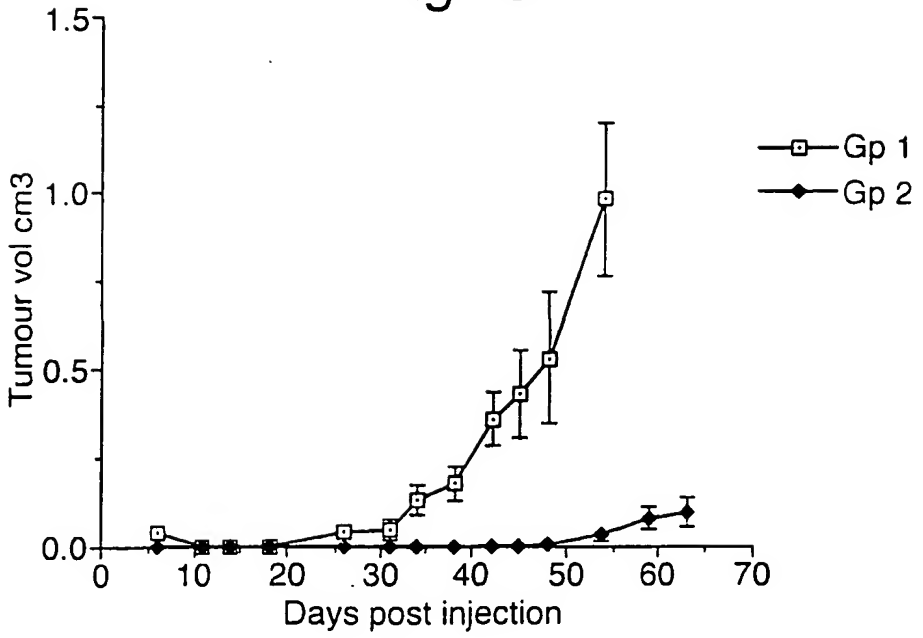


Fig.13.


1.5
1.0
0.5
0.0
Tumour vol cm3
0
10
20
30
40
50
60
70
Days post injection
Gp 1
Gp 2

SEQUENCE LISTING

SEQUENCE DESCRIPTION: SEQ ID NO: 1 and 2, the polynucleotide sequence and amino acid sequence of human Brn-3a, respectively.

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ATG ATG TCC ATG AAC AGC AAG CAG CCT CAC TTT GCC ATG CAT CCC ACC        48
Met Met Ser Met Asn Ser Lys Gln Pro His Phe Ala Met His Pro Thr
 1               5                  10                  15

CTC CCT GAG CAC AAG TAC CCG TCG CTG CAC TCC AGC TCC GAG GCC ATC        96
Leu Pro Glu His Lys Tyr Pro Ser Leu His Ser Ser Ser Glu Ala Ile
            20                  25                  30

CGG CGG GCC TGC CTG CCC ACG CCG CCG CTG CAG AGC AAC CTC TTC GCC       144
Arg Arg Ala Cys Leu Pro Thr Pro Pro Leu Gln Ser Asn Leu Phe Ala
        35                  40                  45

AGC CTG GAC GAG ACG CTG CTG GCG CGG GCC GAG GCG CTG GCG GCC GTG       192
Ser Leu Asp Glu Thr Leu Leu Ala Arg Ala Glu Ala Leu Ala Ala Val
    50                  55                  60

GAC ATC GCC GTG TCC CAG GGC AAG AGC CAT CCT TTC AAG CCG GAC GCC       240
Asp Ile Ala Val Ser Gln Gly Lys Ser His Pro Phe Lys Pro Asp Ala
65                  70                  75                  80

ACG TAC CAC ACG ATG AAC AGC GTG CCG TGC ACG TCC ACT TCC ACG GTG       288
Thr Tyr His Thr Met Asn Ser Val Pro Cys Thr Ser Thr Ser Thr Val
                85                  90                  95

CCT CTG CGG CAC CAC CAC CAC CAC CAC CAC CAC CAC CAG GCG CTC GAA       336
Pro Leu Arg His His His His His His His His His Gln Ala Leu Glu
            100                 105                 110

CCC GGC GAT CTG CTG GAC CAC ATC TCC TCG CCG TCG CTC GCG CTC ATG       384
Pro Gly Asp Leu Leu Asp His Ile Ser Ser Pro Ser Leu Ala Leu Met
        115                 120                 125

GCC GGC GCG GGC GGC GCG GGC GGC GCG GGC GCG GCC GGC GGC GGC       432
Ala Gly Ala Gly Gly Ala Gly Gly Ala Gly Ala Ala Ala Gly Gly Gly
    130                 135                 140
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```
GGC GCC CAC GAC GGC CCG GGG GGC GGT GGC GGC CCG GGC GGC GGC GGC       480
Gly Ala His Asp Gly Pro Gly Gly Gly Gly Gly Pro Gly Gly Gly Gly
145                 150                 155                 160
GGC CCG GGC GGC GGC GGC CCC GGG GGA GGC GGC GGT GGC GGC CCG GGG       528
Gly Pro Gly Gly Gly Gly Pro Gly Gly Gly Gly Gly Gly Gly Pro Gly
                165                 170                 175
GGC GGC GGC GGC GGC CCG GGC GGC GGG CTC CTG GGC GGC TCC GCG CAC       576
Gly Gly Gly Gly Gly Pro Gly Gly Gly Leu Leu Gly Gly Ser Ala His
            180                 185                 190
CCT CAC CCG CAT ATG CAC AGC CTG GGC CAC CTG TCG CAC CCC GCG GCG       624
Pro His Pro His Met His Ser Leu Gly His Leu Ser His Pro Ala Ala
        195                 200                 205
GCG GCC GCC ATG AAC ATG CCG TCC GGG CTG CCG CAC CCC GGG CTG GTG       672
Ala Ala Ala Met Asn Met Pro Ser Gly Leu Pro His Pro Gly Leu Val
    210                 215                 220
GCG GCG GCG GCG CAC CAC GGC GCG GCA GCG GCA GCG GCG GCG GCG GCG       720
Ala Ala Ala Ala His His Gly Ala Ala Ala Ala Ala Ala Ala Ala Ala
225                 230                 235                 240
GCC GGG CAG GTG GCA GCG GCA TCG GCG GCG GCG GCC GTG GTG GGC GCA       768
Ala Gly Gln Val Ala Ala Ala Ser Ala Ala Ala Ala Val Val Gly Ala
                245                 250                 255
GCG GGC CTG GCG TCC ATC TGC GAC TCG GAC ACG GAC CCG CGC GAG CTC       816
Ala Gly Leu Ala Ser Ile Cys Asp Ser Asp Thr Asp Pro Arg Glu Leu
            260                 265                 270
GAG GCG TTC GCG GAG CGC TTC AAG CAG CGG CGC ATC AAG CTG GGC GTG       864
Glu Ala Phe Ala Glu Arg Phe Lys Gln Arg Arg Ile Lys Leu Gly Val
        275                 280                 285
ACG CAG GCC GAC GTG GGC TCG GCG CTG GCC AAC CTC AAG ATC CCG GGC       912
Thr Gln Ala Asp Val Gly Ser Ala Leu Ala Asn Leu Lys Ile Pro Gly
    290                 295                 300
```

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Val Gly Ser Leu Ser Gln Ser Thr Ile Cys Arg Phe Glu Ser Leu Thr
305                 310                 315                 320
CTC TCG CAC AAC AAC ATG ATC GCG CTC AAG CCC ATC CTG CAG GCG TGG     1008
Leu Ser His Asn Asn Met Ile Ala Leu Lys Pro Ile Leu Gln Ala Trp
                325                 330                 335
CTC GAG GAG GCC GAG GGC GCC CAG CGC GAG AAA ATG AAC AAG CCT GAG     1056
Leu Glu Glu Ala Glu Gly Ala Gln Arg Glu Lys Met Asn Lys Pro Glu
            340                 345                 350
CTC TTC AAC GGC GGC GAG AAG AAG CGC AAG CGG ACT TCC ATC GCC GCG     1104
Leu Phe Asn Gly Gly Glu Lys Lys Arg Lys Arg Thr Ser Ile Ala Ala
        355                 360                 365
CCC GAG AAG CGC TCC CTC GAG GCC TAC TTC GCC GTG CAG CCC CGG CCC     1152
Pro Glu Lys Arg Ser Leu Glu Ala Tyr Phe Ala Val Gln Pro Arg Pro
    370                 375                 380
TCG TCC GAG AAG ATC GCC GCC ATC GCC GAG AAA CTG GAC CTC AAA AAG     1200
Ser Ser Glu Lys Ile Ala Ala Ile Ala Glu Lys Leu Asp Leu Lys Lys
385                 390                 395                 400
AAC GTG GTG CGG GTG TGG TTT TGC AAC CAG AGA CAG AAG CAG AAG CGG     1248
Asn Val Val Arg Val Trp Phe Cys Asn Gln Arg Gln Lys Gln Lys Arg
                405                 410                 415
ATG AAA TTC TCT GCC ACT TAC TGA                                    1272
Met Lys Phe Ser Ala Thr Tyr  *
            420
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SEQUENCE DESCRIPTION: SEQ ID NO: 3 and 4, the polynucleotide and amino acid sequence of mouse Brn-3a, respectively

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ATG ATG TCC ATG AAC AGC AAG CAG CCT CAC TTT GCC ATG CAT CCC ACC      48
Met Met Ser Met Asn Ser Lys Gln Pro His Phe Ala Met His Pro Thr
425                 430                 435                 440
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CTC CCT GAG CAC AAG TAC CCG TCG CTG CAC TCC AGC TCC GAG GCC ATC        96
Leu Pro Glu His Lys Tyr Pro Ser Leu His Ser Ser Ser Glu Ala Ile
                445                 450                 455
CGG CGG GCC TGC CTG CCC ACG CCG CCG CTG CAG AGC AAC CTC TTC GCC       144
Arg Arg Ala Cys Leu Pro Thr Pro Pro Leu Gln Ser Asn Leu Phe Ala
            460                 465                 470
AGC CTG GAC GAG ACG CTG CTG GCG CGG GCC GAG GCG CTG GCG GCC GTG       192
Ser Leu Asp Glu Thr Leu Leu Ala Arg Ala Glu Ala Leu Ala Ala Val
        475                 480                 485
GAC ATC GCG GTG TCC CAG GGC AAG AGC CAC CCT TTC AAG CCG GAC GCC       240
Asp Ile Ala Val Ser Gln Gly Lys Ser His Pro Phe Lys Pro Asp Ala
    490                 495                 500
ACG TAC CAC ACG ATG AAT AGC GTG CCC TGC ACG TCC ACG TCC ACC GTG       288
Thr Tyr His Thr Met Asn Ser Val Pro Cys Thr Ser Thr Ser Thr Val
505                 510                 515                 520
CCG CTG GCG CAC CAC CAC CAC CAC CAC CAC CAC CAC CAG GCG CTC GAG       336
Pro Leu Ala His His His His His His His His His Gln Ala Leu Glu
                525                 530                 535
CCC GGT GAC CTG CTG GAC CAC ATC TCG TCG CCG TCG CTC GCG CTC ATG       384
Pro Gly Asp Leu Leu Asp His Ile Ser Ser Pro Ser Leu Ala Leu Met
            540                 545                 550
GCC GGC GCA GGG GGC GCA GGC GCG GCG GGA GGC GGC GGC GGC GCC CAC       432
Ala Gly Ala Gly Gly Ala Gly Ala Ala Gly Gly Gly Gly Gly Ala His
        555                 560                 565
GAC GGC CCC GGG GGC GGA GGC GGA CCG GGG GGC GGC GGT GGC CCG GGC       480
Asp Gly Pro Gly Gly Gly Gly Gly Pro Gly Gly Gly Gly Gly Pro Gly
    570                 575                 580
GGC GGC GGC CCC GGG GGT GGC GGC GGC GGC GGC GGC CCG GGG GGC GGC       528
Gly Gly Gly Pro Gly Gly Gly Gly Gly Gly Gly Gly Pro Gly Gly Gly
585                 590                 595                 600
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GGC GGC GCC CCG GGC GGC GGG CTC TTG GGC GGC TCG GCG CAT CCG CAC 576
Gly Gly Ala Pro Gly Gly Gly Leu Leu Gly Gly Ser Ala His Pro His
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CCG CAC ATG CAC GGC CTG GGC CAC CTG TCG CAC CCC GCG GCG GCG GCG 624
Pro His Met His Gly Leu Gly His Leu Ser His Pro Ala Ala Ala Ala
620 625 630

GCC ATG AAC ATG CCG TCC GGG CTG CCG CAT CCC GGG CTC GTG GCC GCG 672
Ala Met Asn Met Pro Ser Gly Leu Pro His Pro Gly Leu Val Ala Ala
635 640 645

GCG GCG CAC CAC GGC GCG GCG GCG GCA GCG GCG GCG GCG GCG GCG GGG 720
Ala Ala His His Gly Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Gly
650 655 660

CAG GTG GCG GCG GCG TCG GCC GCG GCG GCG GTG GTG GGC GCG GCG GGC 768
Gln Val Ala Ala Ala Ser Ala Ala Ala Ala Val Val Gly Ala Ala Gly
665 670 675 680

CTG GCG TCC ATC TGC GAC TCG GAC ACG GAC CCG CGC GAG CTC GAG GCG 816
Leu Ala Ser Ile Cys Asp Ser Asp Thr Asp Pro Arg Glu Leu Glu Ala
685 690 695

TTC GCC GAG CGC TTC AAG CAG CGG CGC ATC AAG CTG GGC GTG ACG CAG 864
Phe Ala Glu Arg Phe Lys Gln Arg Arg Ile Lys Leu Gly Val Thr Gln
700 705 710

GCC GAC GTG GGC TCG GCG CTG GCC AAC CTC AAG ATC CCG GGC GTG GGC 912
Ala Asp Val Gly Ser Ala Leu Ala Asn Leu Lys Ile Pro Gly Val Gly
715 720 725

TCG CTC AGC CAG AGC ACC ATC TGC AGG TTC GAG TCG CTC ACG CTC TCG 960
Ser Leu Ser Gln Ser Thr Ile Cys Arg Phe Glu Ser Leu Thr Leu Ser
730 735 740

CAC AAC AAC ATG ATC GCG CTC AAG CCC ATC CTG CAG GCG TGG CTG GAG 1008
His Asn Asn Met Ile Ala Leu Lys Pro Ile Leu Gln Ala Trp Leu Glu
745 750 755 760

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GAG GCC GAG GGC GCG CAG CGT GAG AAA ATG AAC AAG CCG GAG CTC TTC      1056
Glu Ala Glu Gly Ala Gln Arg Glu Lys Met Asn Lys Pro Glu Leu Phe
                765                 770                 775

AAC GGC GGC GAG AAG AAG CGC AAG CGG ACT TCC ATC GCC GCG CCC GAG      1104
Asn Gly Gly Glu Lys Lys Arg Lys Arg Thr Ser Ile Ala Ala Pro Glu
            780                 785                 790

AAG CGC TCC CTC GAG GCC TAT TTT GCC GTA CAA CCC CGG CCC TCG TCT      1152
Lys Arg Ser Leu Glu Ala Tyr Phe Ala Val Gln Pro Arg Pro Ser Ser
        795                 800                 805

GAG AAG ATC GCC GCC ATC GCC GAG AAA CTG GAC CTC AAA AAG AAC GTG      1200
Glu Lys Ile Ala Ala Ile Ala Glu Lys Leu Asp Leu Lys Lys Asn Val
    810                 815                 820

GTG CGG GTG TGG TTT TGC AAC CAG AGA CAG AAG CAG AAG CGG ATG AAA      1248
Val Arg Val Trp Phe Cys Asn Gln Arg Gln Lys Gln Lys Arg Met Lys  825
        830                 835                 840

TTC TCT GCC ACT TAC TGA                                             1266
Phe Ser Ala Thr Tyr  *
                845
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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 99/04116

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12Q1/70 C07K14/47 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NDISDANG DANIEL ET AL: "The HPV-activating cellular transcription factor Brn-3a is overepressed in CIN3 cervical lesions." JOURNAL OF CLINICAL INVESTIGATION APRIL 15, 1998, vol. 101, no. 8, 15 April 1998 (1998-04-15), pages 1687-1692, XP000867063 ISSN: 0021-9738 cited in the application the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents .

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 99/04116

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	THEIL THOMAS ET AL: "Short isoform of POU factor Brn-3b can form a heterodimer with Brn-3a that is inactive for octamer motif binding." JOURNAL OF BIOLOGICAL CHEMISTRY 1995, vol. 270, no. 52, 1995, pages 30958-30964, XP002135722 ISSN: 0021-9258 the whole document ---	1-9,13
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US1994 MORRIS PETER J ET AL: "The opposite and antagonistic effects of the closely related POU family transcription factors Brn-3a and Brn-3b on the activity of a target promoter are dependent on differences in the POU domain." Database accession no. PREV199497544582 XP002135724 cited in the application abstract & MOLECULAR AND CELLULAR BIOLOGY 1994, vol. 14, no. 10, 1994, pages 6907-6914, ISSN: 0270-7306 ---	1-9,13
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US1997 LATCHMAN DAVID S: "Regulation of neuroblastoma growth and differentiation by the POU family transcription factors Brn-3a and Brn-3b (review)." Database accession no. PREV199799597457 XP002135725 abstract & INTERNATIONAL JOURNAL OF ONCOLOGY 1997, vol. 10, no. 6, 1997, pages 1133-1139, ISSN: 1019-6439 ---	1-9,13
A	SMITH ET AL: "The Brn-3a transcription factor induces neuronal proceses outgrowth and the coordinate expression of genes encoding synaptic proteins" MOLECULAR AND CELLULAR BIOLOGY,US,WASHINGTON, DC, vol. 17, no. 1, 1 January 1997 (1997-01-01), pages 345-354, XP002087059 ISSN: 0270-7306 --- -/--	

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/GB 99/04116

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 05272 A (SMITH MARTIN DAMIAN ;UNIV LONDON (GB); LATCHMAN DAVID SEYMOUR (GB)) 4 February 1999 (1999-02-04) the whole document ---	1-16
P,X	NDISANG DANIEL ET AL: "The Brn-3a transcription factor plays a critical role in regulating human papilloma virus gene expression and determining the growth characteristics of cervical cancer cells." JOURNAL OF BIOLOGICAL CHEMISTRY OCT. 1, 1999, vol. 274, no. 40, 1 October 1999 (1999-10-01), pages 28521-28527, XP002135723 ISSN: 0021-9258 the whole document -----	1-16

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/GB 99/04116

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9905272 A	04-02-1999	AU 8548198 A	16-02-1999